DIAGNOSTIC TESTING FOR SWINE RESPIRATORY VIRUSES USING ORAL FLUIDS IN SASKATCHEWAN

A thesis submitted to the college of Graduate Studies and Research in partial fulfillment of the requirements for the degree Master of Science in the department of Veterinary Pathology University of Saskatchewan, Saskatoon.

By

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Abstract

Porcine respiratory disease complex (PRDC) is one of the most economically significant clinical conditions in growing pigs worldwide. The clinical signs of PRDC are typical for respiratory diseases and the most common pathogens that play a role in PRDC are: porcine reproductive and respiratory disease syndrome virus (PRRSV), influenza A virus (IAV-S) and porcine circovirus type 2 (PCV2). The primary purpose of this study is to assess the detection frequency of these three viruses on selected farms within Saskatchewan using oral fluids (OF). Additional purposes are to examine the monthly trends of each of the three respiratory viruses, to evaluate the positive frequency of OF and NSSW by sample, barn and month in grower pigs for IAV-S detection. Furthermore, the purpose was to assess the ability to detect the specific genetic types of IAV-S strains circulating in SK while comparing results of IAV-S testing for OF and NSSW samples. Validation of real-time PCR procedures for PRRSV, IAV-S, and PCV2, was conducted using ten-fold dilution series within OF and sterile water with each virus. The amplification efficiencies for manual and machine extraction of these dilutions for PRRSV, IAV-S, and PCV2, were calculated. For the main project, we collected six OF samples per month from each of ten and nine grower- finisher farms for five consecutive months over the winters of 2013-2014 and 2014-2015, respectively (509 samples total). Real-time PCR was used to detect viral nucleic acid in all OF samples. Barn-level prevalence was similar between the two years for all three pathogens. However, detection frequency of three pathogens changed over the period of collection, and the pathogens detected varied month-to-month. Furthermore, more than one pathogen was found in six farms, and all three pathogens were found in one farm in both years. NSSW received with some of the OF samples were tested for IAV-S and detection of IAV-S RNA was more frequent with NSSW than OF. In conclusion, NSSW has a high positive frequency for IAV-S detection compared to OF by sample, barn and month. Moreover, there was

no effect of the season for sampling and currently circulating influenza viruses of these Saskatchewan swine farms are alpha H1N1, 2009 pandemic H1N1, and Cluster IV H3N2.

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Dedication

To my mother and father

For all of the sacrifices that you've made on my behalf

To my husband Nelanka

For his speechless support and love

Original contribution

This thesis presents the findings of an independent research project. I contributed extensively to all aspects of this research project, from project design to final presentation. Funding was used for the purchase of laboratory utensils and materials associated with the research. My responsibilities included performing all laboratory work associated with a research project. Prairie Diagnostic Services, Inc. (PDS) provided all laboratory facilities. University of Minnesota Veterinary Diagnostic Laboratory performed the virus isolation and sequencing of influenza A virus.

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List of abbreviations

°C	Degrees Celsius
BALF	Bronchoalveolar Lavage Fluid
CFIA	Canadian Food Inspection Agency
cm	Centimeter
CPE	Cytopathic Effects
Ct	Cycle threshold
DNA	Deoxyribonucleic Acid
ELISA	Enzyme Linked Immunosorbent Assay
HA	Hemagglutination Assay
HI	Hemagglutination Inhibition Assay
IFA	Immunofluorescence Assay
IAV-S	Influenza A virus in swine
IgA	Immunoglobulin A
IgM	Immunoglobulin M
IgG	Immunoglobulin G
IHC	Immunohistochemistry
M	Matrix
MDA	Maternally Derived Antibodies
MDCK	Madin-Darby Canine Kidney
Mhyo	*Mycoplasma hyopneumoniae*
ml	Milliliter
NA	Neuraminidase

NP	Nucleoprotein
ORF	Open reading frame
PCR	Polymerase Chain Reaction
PCV1	Porcine Circovirus type 1
PCV2	Porcine Circovirus type 2
PDS	Prairie Diagnostic Services, Inc.
PRCV	Porcine Respiratory Corona virus
PRDC	Porcine Respiratory Disease Complex
PRRS	Porcine Reproductive and Respiratory Syndrome
PRRSV	Porcine Reproductive and Respiratory Syndrome virus
PRV	Pseudorabies virus
qPCR	quantitative Polymerase Chain Reaction
RNA	Ribonucleic Acid
RRT-PCR	Real-Time Reverse Transcription Polymerase Chain Reaction
RT-PCR	Reverse Transcription Polymerase Chain Reaction
SK	Saskatchewan
SN	Serum Neutralization
$TCID_{50}$	Median Tissue Culture Infective Dose
TX98	Influenza A/swine/Texas/4299-2/1998 H3N2
USA	United States of America
UMVDL	University of Minnesota, Veterinary Diagnostic Laboratory
µl	Microliter
µm	Micrometer

1 INTRODUCTION AND LITERATURE REVIEW

1.1 General Introduction

Respiratory diseases are a major cause economic losses in swine industry (Opriessing 2011) due to their high prevalence. These losses often come from low weight gain, high mortality rate, carcass condemnation at slaughter, high feed cost due to higher age at slaughter, costs for treatments and vaccines and maintenance and labor cost.

Porcine respiratory disease complex (PRDC) is one of the most prevalent and economically significant swine clinical conditions worldwide (Opriessnig 2011). The epidemiology of PRDC can vary among different countries, geographical areas and farms. Therefore, it is difficult to obtain the exact prevalence of PRDC on a global scale. However, in the literature, there are a number of published data about mixed respiratory infections of swine in the USA, Canada, Switzerland, Belgium and Canada (Hansen 2010).

The viral PRDC pathogens that will be discussed in this thesis include porcine reproductive and respiratory disease syndrome virus (PRRSV), influenza A virus in swine (IAV-S) and porcine circovirus type 2 (PCV2).

1.2 PRDC

The disease complex is multi-factorial, with both infectious and non-infectious factors contributing to disease (Opriessnig 2011). It is predominantly seen in grower-finisher pigs between the ages of three and six months, and the clinical signs of PRDC are typical for respiratory diseases and include: anorexia, fever, cough, dyspnea, lethargy and decreased growth rate (Opriessnig 2011). The disease complex is characterized by 30-70% morbidity and 4-6% mortality (Opriessnig 2011, Gimenez-Lirola 2011)

Typically two or more viral, bacterial or parasitic pathogens affect an individual concurrently, making PRDC polymicrobial in nature (Opriessnig 2011). They are considered as primary or

secondary pathogens based on the capability of inducing disease. Primary pathogens can induce the disease by their own virulence. The most common primary viral pathogens are: PRRSV, IAV-S, PCV2, pseudorabies virus (PRV), and porcine respiratory coronavirus (PRCV). The most common primary bacterial pathogens are: *Mycoplasma hyopneumoniae* (Mhyo), *Bordetella bronchiseptica,* and *Actinobacillus pleuropneumoniae* (Sibila 2009). However, secondary pathogens invade the host opportunistically after primary pathogens have caused damage to the tissues and innate defenses (Opriessing 2011). The common opportunistic agents are *Pasteurella multocida, Haemophilus parasuis, Streptococcus suis, Actinobacillus suis, Trueperella pyogenes (Arcanobacterium pyogenes),* and *Salmonella choleraesuis* (Sibila 2009).

As a multi-factorial condition, PRDC is influenced by non-infectious factors including: the environment, population size, management strategies and host responses (Opriessing 2011). In fact, dramatic temperature changes, suboptimal indoor air quality and high levels of ammonia may act as unfavorable conditions. Furthermore, inadequate or poor quality feed and water supply, high population density, and other stress factors play a major role in causing PRDC (Opriessing 2011). Additionally, age, immunity, and the genetic background of the animal are important factors. One of these factors or many combinations of factors can predispose to initiate the disease complex with the invasion of infectious agents (Brockmeier 2002).

1.3 Porcine reproductive and respiratory syndrome virus

Porcine reproductive and respiratory syndrome (PRRS) is a chronic, persistent respiratory disease of pigs (Terpstra 1991) of any age caused by PRRSV. Originally PRRS was termed "mystery pig disease" (Goyal 1993) however, this mystery disease was originally thought to be caused by encephalomyocarditis (EMC) virus (Joo 1988). PRRS was first described in the United States in 1987 in pigs with the clinical signs of reduced appetite, reproductive problems

including abortions, mummified fetuses, stillbirths, infertility, and postpartum respiratory problems (Hill 1990). In 1991, farmers in Netherlands observed similar clinical signs in their swine and since the late 1990s, it was identified in many European countries including Germany, Denmark, and the United Kingdom. In the early 1990s similar outbreaks occurred in Japan and Taiwan as well (Chang 1993).

PRRSV is one of the most important causative agents of PRDC, and it can be difficult to control. PRRSV is endemic in many countries; however, Finland, Sweden, Norway, Switzerland, New Caledonia, New Zealand, Australia, Argentina, and Cuba are a few countries fortunate to be free of PRRSV (Zimmerman 2012). Moreover, it contributes the highest production losses compared to other respiratory diseases (Chand 2012, Linhares 2012, Opriessnig 2011), resulting in losses of approximately $664 million (USD) for growing pigs in North America alone (Holtkamp 2013).

1.3.1 Porcine Reproductive and respiratory syndrome virus virology

In 1991, Dutch scientists had identified the etiological agent and named it the Lelystad virus (Wensvoort 1991, Terpstra 1991). At the same time, U.S. scientists had isolated the virus causing swine infertility and a respiratory syndrome that was designated as ATCC VR-2332 (Benfield 1992). The virus was officially named PRRSV in 1991 (Terpstra 1991). However, since the 1980s, PRRS still causes uncontrollable clinical outbreaks and huge economic impact worldwide.

PRRSV is a positive-sense, single-stranded, enveloped RNA virus (Chand 2012). It belongs to order Nidovirales and family Arteriviridae and classified with equine arteritis virus (EAV), lactate dehydrogenase-elevating virus (LDV), and simian hemorrhagic fever virus. According to 44% nucleotide changes in the genome, PRRSV has two major lineages. Type 1 PRRSV are

from the European lineage with the prototype Lelystad virus (LV), and the North American lineage is type 2 with prototype VR2332. Both genotypes currently have worldwide distribution. More recently, the type 3 PRRSV emerged in Europe with the first isolate and prototype virus designated Lena virus (Karniychuk 2010). This highly pathogenic Lena virus is genetically and antigenically different from European subtype Lelystad virus and North American US5 strains (Karniychuk 2010).

PRRSV has a 15.4 kb long genome that has ten open reading frames or ORFs (Chand 2012). ORF 5, 6 and 7 code for envelope (GP 5), membrane (M) and nucleocapsid (N) proteins, respectively that are the major structural proteins of the virus (Meulenberg 1995). Due to high variations and external position of the envelope (GP 5) protein, it is often used for phylogenetic analyses of PRRSV (Zimmerman 2012).

In 2006, China experienced the high pathogenic PRRSV outbreak caused by a PRRSV strain with a unique 30-amino-acid (30-aa) discontinuous deletion in its Nsp2-coding region (Zhou 2009). According to Zhou 2009, the Nsp2-coding region with deletion is not related to its virulence. Hence, the authors suggested that this region could be used to distinguish highly pathogenic Chinese strain from North American strains.

1.3.2 PRRSV pathogenesis

PRRSV-infected swine shed the virus through a number of routes such as nasal aerosols and nasal secretions (Christianson 1993, Rossow 1995), saliva (Wills 1997), urine (Wills 1997), semen (Swenson 1994), milk (Wagstrom 2001) and feces (Christianson 1993). The virus can be transmitted through several routes including intranasal, oral, intramuscular, intrauterine, venereal and direct contact with contaminated fomites. PRRSV transmits from an infected dam to her litter efficiently during the third trimester of the pregnancy.

Once a pig is infected with PRRSV, the virus primarily targets alveolar macrophages in the lungs, but also macrophages of other tissues. The severity of the infection depends on the virulence of the PRRSV variant (Johnson 2004). Virulence of PRRSV is influenced by host age, breed, gender, immune status and the virus strain (Wensvoort 1993).

Feral swine are seldom suspected as a source, and the original source of the virus is still unknown. Transmission of PRRSV by flies has been observed and flies act as fomites. However, carrier animals are responsible for causing persistent infection within herds. The virus can stay stable for a long time if the environment is favorable and surprisingly though the mechanism is unclear, it is capable of spreading through the air for distances of at least 9.2 km (Otake 2010).

1.3.3 PRRS clinical disease

PRRS is clinically characterized by both reproductive and respiratory problems in swine. The clinical presentation may vary with the virulence of the strain, herd immunity, host genetic background and management factors. Less virulent variants often produce subclinical infections, and highly virulent variants produce severe disease (Johnson 2004). Affected pigs may show either reproductive or respiratory signs, or both, depending on which production groups are present on the farm.

Reproductive problems are more often experienced in female pigs compared with male pigs and initially they show anorexia, lethargy and later reproductive failure. In addition to late term abortions, more commonly seen are litters with a range of dead fetuses, weak-born and apparently healthy piglets. Since fetuses may die over several days or weeks, there can be dead fetuses of varying size and stages of post-mortem autolysis present (Wensvoort 1993). Infected boars show anorexia, lethargy, poor reproductive performance with reduced libido, semen quality and abnormal sperm morphologies (Feitsma 1992).

Respiratory problems can occur at any age, but nursery and growing pigs are more susceptible than adults to the respiratory syndrome of PRRS. The common clinical outcomes of the respiratory manifestation of PRRSV infection are lethargy, inappetence, rough hair, dyspnea and reddish-blue discoloration of ears or hind quarters (Wensvoort 1993).

1.3.4 PRRS pathologic lesions

Gross and microscopic lesions in growing pigs are most often seen in lungs and lymph nodes. The severity of these lesions depends on the virulence of the virus, as well as several host factors. The principal lesion of interstitial pneumonia results in lungs that are slightly firm, moist, non-collapsing and mottled gray to tan in color (Dietze 2011). Microscopically, the alveolar and interstitial septae are expanded 10 to 20 times the normal width and increased numbers of macrophages are seen within the septae and alveolar spaces. Occasionally, apoptosis of type1 pneumocytes and alveolar macrophages is also seen.

1.3.5 PRRSV diagnostics

Diagnosis of PRRS is based on clinical history, production status, clinical signs, necropsy findings and laboratory results. Both clinical and subclinical conditions decrease productivity level by reducing the fertility and health condition of swine. Detection of the pathogen, PRRSV, is one of the key factors in diagnosing PRRS.

Diagnostic samples for PRRSV include: serum, tissues from lung, lymph nodes, stillborn piglets, mummified fetuses, semen (Zimmerman 2012) and oral fluids (Chittick 2011, Prickett 2008). The choice of sample to be collected will be determined by the choice of laboratory techniques and vice versa. The virus can be isolated from tissue homogenates or serum using porcine alveolar macrophage (PAM) (OIE 2013) and MARC- 145 cells (Dietze 2011). Antibodies are mainly detected by an enzyme-linked immunosorbent assay (ELISA) technique in serum, but

oral fluids can also be used with ELISA (Detmer 2013a). Virus antigens are often visualized by immunohistochemistry (IHC) in tissue (Dietze 2011), but an immunofluorescence (IFA) assay (Yoon 1995) can also be used to detect antibodies in serum. Reverse transcription PCR (RT-PCR) is highly sensitive for detection of viral ribonucleic acids (RNA), but it cannot discern the live virus from dead virus. The most sensitive and common methods of detection for PRRSV are real-time RT-PCR (RRT-PCR) and quantitative RT-PCR (RT-qPCR).

1.3.6 Prevention and control of PRRS

Prevention and control strategies are often based on improving biosecurity protocols and herd immunity, depopulation of herds and vaccination programs (OIE 2013). Commonly used biosecurity measures are the quarantine of new animals, proper sanitation methods, disinfection of fomites and maintenance of air filtration systems (OIE 2013). Herd immunity can be improved by vaccination and exposure of susceptible herds to infected animals and contaminated fomites (Zimmerman 2012). Furthermore, modified live viral vaccines and killed vaccines are commercially available, and they reduce the clinical disease and the transmission of wild-type virus. However, modified-live viral vaccines are not recommended for use in naïve herds, pregnant females, and breeding animals because it causes infection and circulation of PRRSV in the herd (Botner 1997). Additionally, modified live viral vaccines have the capability of reverting to more virulent forms and cause herd outbreaks (Dietze 2011).

1.4 Influenza A virus in swine

Influenza A virus (IAV) is a common viral disease among humans and animals. It causes acute respiratory conditions that are characterized by lethargy, pyrexia, loss of appetite, respiratory distress and nasal discharges (Janke 2014). The severity of the disease depends on the viral

strain, the immune status, and other predisposing factors. Pigs are susceptible to influenza type A and occasionally influenza type C viruses (Vincent 2008).

IAV in swine (IAV-S) is a highly contagious and it is an important etiological agent of PRDC (Opriessnig 2011). Additionally, IAV-S is a zoonotic agent associated with human-to-pig and pig-to-human transmissions. The best example of this is the 2009 pandemic virus (pH1N1) that is endemic in both human and pig populations (Nelson 2015a). Less often, there are human viruses that transmit to pigs (Nelson 2012, Nelson 2015a) and swine viruses that transmit to people (Myers 2007).

1.4.1 IAV-S virology

Influenza viruses belong to Family *Orthomyxoviridae* and are classified by the type, subtype, and genotype (Lamb 2013). Family *Orthomyxoviridae* contains influenza A, B and C genera. Influenza A is a true zoonotic agent and B and C are mainly human pathogens. However, swine and dogs are susceptible to influenza C. IAV-S are further divided according to the hemagglutinin (HA) and neuraminidase (NA) surface proteins (Vincent 2008), and there are 16 HA and 9 NA IAV subtypes have been found in aquatic birds (Webster 2006). H17N10 and H18N11 have been found in bats (Tong 2012).

IAV-S was first recognized in the Midwestern United States (Myers 2007) concurrently with "Spanish flu" that was a devastating human influenza pandemic in 1918 (Webster 2002). The genetic evolutionary analysis shows that the 1918 pandemic strain is the likely ancestor (Reid 1999) to the first IAV-S isolated in 1930, an H1N1virus (Shope 1931). Both this virus and the 1918 pandemic strain have been determined to be of avian origin (Taubenberger 2006). In 1977, US scientists had isolated human H3N2 virus from pigs in Colorado (Karasin 2000). However,

until late 1990s H1N1 was the predominant influenza subtype in North American swine and it termed as classical swine H1N1 (cH1N1).

In 1998, the first triple-reassortant H3N2 subtype was isolated in North American swine containing gene segments from human, pig and avian viruses (Webby 2000, Lorusso 2011). As the triple-reassortant H3N2 became endemic, H1N1 viruses acquired the triple reassortant internal gene cassette (TRIG) and a number of novel influenza subtypes like H1N2, and H1N1 viruses (Olsen 2002, Vincent 2008), as well as wholly avian H4N6, H3N3, and H1N1 swine isolates (Karasin 2000) emerged. The predominant subtypes currently found in pigs are H1N1, H1N2, and H3N2.

The negative-sense, single-stranded and segmented RNA genome has eight RNA segments: polymerase basic 2 (PB2), polymerase basic 1 (PB1), polymerase acidic (PA), hemagglutinin (HA), nucleoprotein (NP) neuraminidase (NA), matrix (M) and nonstructural (NS) (Fields 2001). The components of the TRIG cassette are swine-origin M, NS, and NP, avian-origin PA and PB2, and human-origin PB1.

The surface glycoproteins, HA, and NA are highly susceptible to mutations and show the highest genetic diversity. Antigenic drift caused by the accumulation of point mutations and antigenic shift caused by reassortment of gene segments has brought about the recognition of five distinct genetic clusters within the H1 subtype: α, β, γ, δ1 and δ2 (Lorusso 2011). The TRIG cassette appears to be responsible for the ability of the North American swine viruses to pick up new HA and NA genes resulting in an explosion in IAV-S evolution (Lorusso 2011).

1.4.2 IAV-S pathogenesis

The virus replicates in epithelial cells of both lower and upper respiratory tract of swine, and a larger viral load can be seen in lung tissues than other parts of the respiratory tract (Janke 2014).

IAVs bind to sialic acid receptors on the surface of epithelial cells. It is generally accepted that human and swine IAVs bind to α2,6 – sialic acid receptors, whereas avian IAVs bind to α2,3 – sialic acid receptors. Deep within the respiratory tract of pigs and humans, both α2,6 and α2,3 receptors are present on epithelial cells, which is why both species can be infected by human, swine and avian IAVs (Palese 2004). Although pigs are susceptible to human and avian IAVs, binding with sialic acid receptor may not be enough to cause an infection (Bateman 2013) due to the complex nature of the receptor specificity (Bateman 2013), which may explain why zoonosis in humans and pigs is uncommon (Myers 2007, Nelson 2015b). In Europe, the avian-like swine H1N1 virus was identified as a wholly avian-origin virus (Van Reeth 2013). However, due to the spread of pH1N1 in Europe among pigs and people, there are now triple and quadruple reassortant viruses with variations on the TRIG cassette seen in North America (Van Reeth 2013)

1.4.3 IAV-S clinical disease

Within 24 hours of infection, pigs develop pyrexia and initiate virus excretion. IAV-S is found in nasal secretions, tracheal and bronchial fluid, lung and oral fluids (Detmer 2011, Schultz-Cherry 2013). The peak of virus excretion occurs at approximately the same time as the peak of fever and then decreases rapidly. The incubation period of the disease is 1 to 3 days after onset of the infection and rapid recovery occurs 4-7 days of infection. Seroconversion occurs approximately 10 to 14 days after infection and IAV-S antibodies can be detected in the blood (Detmer 2013a).

The respiratory disease is characterized by lethargy, pyrexia, loss of appetite, respiratory distress, cough and nasal discharges (OIE 2013, Schultz-Cherry 2013). The disease has a high morbidity (up to 100%) and comparatively low mortality rate (< 1%) in swine (Vincent 2008). On the other hand, most IAV-S infections caused by less virulent strains are subclinical and not detected by

surveillance programs (Bowman 2012). A number of factors determine the severity of disease including: immune status, age, nutrition, secondary bacterial infections, climate changes and management practices (Janke 2014).

1.4.4 IAV-S pathologic lesions

Macroscopically, lesions are mainly those of viral pneumonia and characterized by bronchointerstitial pneumonia (Olsen 2006). Cranioventrally, lungs display clearly demarcated lobules with purple-red consolidation (Janke 2014). However, these lesions may be enhanced or masked by co-infections with other PRDC pathogens and secondary bacterial infections (Janke 2014).

Microscopically, airways can be filled with necrotic and sloughed bronchiolar epithelial cells, necrotic pneumocytes, cellular debris, proteinaceous fluid and few leukocytes (Janke 2014). Later in infection (7-14 days), peribronchial and perivascular infiltrations of lymphocytes can be seen (Richt 2003).

1.4.5 IAV-S diagnostics

Clinical signs, macroscopic and microscopic lesions, can be used for presumptive diagnosis, but there are no pathognomonic signs to differentiate IAV-S from other respiratory diseases of swine without detection of the virus (Van Reeth 2012). Also, detection of IAV-S is only the first step in diagnosis and treatment of IAV-S. The genetic variability of viruses in circulation necessitates additional procedures, such as subtyping and sequencing, to determine the type of virus present. There are a number of issues to consider for diagnosis of IAV-S: sample timing, sample selection, assay selection, and determination of the genetic and antigenic type of the virus present.

Sample timing is important for detection of the virus within a herd. The short incubation and

recovery period for IAV-S means that there is a small window for detecting IAV-S in a herd. Since coughing usually occurs 7-14 days after infection and peak virus excretion occurs within 24-48 hours of infection (Detmer 2013a), coughing is not a good criterion to select pigs to sample (Janke 2014). Rather, it is recommended to collect samples from untreated swine with elevated rectal temperature within 24 to 72 hours after clinical signs appear (OIE 2015). However, since this is not always practical, the non-coughing, less active pigs who show less interest of feed are more likely to provide a positive sample than the more active coughing pig.

Nasal swabs are the gold standard antemortem sample and lungs are the gold standard post-mortem sample (OIE 2013), but other respiratory samples that can be collected post-mortem include: bronchoalveolar lavage fluid (BALF), nasal turbinates, and trachea (Detmer 2013a). All of these samples can be tested individually or as pools, and it is recommended that several samples (at least three lung samples or at least ten nasal swabs) are tested to increase the chance of viral detection (Detmer 2013a).

For detection of IAV-S, there are several direct and indirect methods available (Detmer 2013a). The viral antigens can be directly identified in clinical samples by IHC, IFA or ELISA techniques, while more direct detection methods, such as RRT-PCR to detect viral RNA or isolation of the whole virus, can also be done (OIE 2015). The most sensitive and common method of detection of IAV-S is RRT-PCR. However, unless there is enough viral RNA in the sample to conduct sequencing, virus isolation is still a common practice using either embryonated chicken eggs or Madin-Darby Canine Kidney (MDCK) cell cultures (Detmer 2013a).

Indirect detection using serological methods is principally done by hemagglutination inhibition (HI) and ELISA, but serum neutralization (SN) is also done by some laboratories (Detmer

2013a). It is recommended that these serological tests can be conducted for paired sera that are collected 14 to 28 days apart. This serum collection would be before (or at the onset of) and after the introduction of a new virus or vaccine to a herd. Paired sera will show increased antibody titers post exposure; usually, the increase is greater than a four-fold increment in antibody titer. However, interpretation of serological analyses is often complicated due to the cross-reactivity of strains within the same subtype (Leuwerke 2008). HI and neuraminidase inhibition (NI) tests can be used for virus subtyping, but it is more common to use subtype-specific RRT-PCR assays (OIE 2015).

Sequencing is most commonly performed on the HA gene due to its high genetic variability and consistent phylogenetic clustering patterns. NA and M are also used to look at evolution patterns in a region, but since pH1N1 introduced new internal genes to the North American viruses, the whole genome is more useful for examining the evolutionary dynamics of IAV-S (Nelson 2015c).

1.4.6 IAV-S prevention and control

As with other respiratory conditions, prevention and control is based on proper management practices and vaccination procedures. Most IAV-S vaccines contain inactivated whole virus. These commercial and farm-specific autogenous vaccines that contain one to four viruses are available throughout North America. However, vector based subunit vaccines are currently only available in the United States (Rajao 2014).

While autogenous vaccines usually provide nearly-complete homologous protection when given to unvaccinated, growing pigs and strong protection when conveyed through the maternally derived antibodies (MDA) following sow vaccination (Allerson 2013, Detmer 2013a, Romagosa 2011), commercial vaccines use highly cross-reactive strains that provide only partial protection

(Detmer 2013a). Nevertheless, the highly unstable nature of the RNA genome of IAVs results in the virus escaping immunity, whichever vaccine is used. Furthermore, the vaccine efficiency can also be blocked by the passive acquired immunity through MDA from the sows (Vincent 2008).

1.5 Type 2 porcine circovirus

PCV2 is another causative agent of PRDC (Opriessnig 2011, Opriessnig 2013). It has a worldwide prevalence and has been detected during outbreaks in North America, South America, Europe and Asia (Chae 2004). PCV2 causes postweaning multisystemic wasting syndrome (PMWS). PMWS is mainly found in 5-12 week old pigs, has a high mortality and relatively low morbidity (Harding 1997). PCV2 is also the cause of porcine dermatitis and nephropathy syndrome (PDNS) (Rosell 2000) and reproductive disorders (West 1999). As a group, these conditions are referred to as porcine circovirus-associated disease (PCVAD) (Opriessnig 2007). The multisystemic involvement and subclinical infections of PCV2 contribute to a large economic loss in the swine industry.

1.5.1 PCV2 virology

Porcine circovirus type 1 (PCV1) was first identified as a contaminant of porcine kidney (PK-15) continuous cell cultures in 1974 (Tischer 1982, Tischer 1974). It was not cytopathogenic for PK-15 cells and not considered a pathogen for swine (Allan. 1995; Tischer 1987). However, in the late 1990s, a new subspecies of circovirus, PCV2, was detected (Allan 1998; Ellis 1998). PCV2 was initially identified in Western Canada among specific pathogen free swine in 1996 (Harding 1996) but was later identified in herds in Alberta, Saskatchewan, Manitoba and Quebec (Harding, 2007, Harding 1998). This new virus subspecies produced a clinical disease in swine, fulfilling Koch`s postulates, and was directly associated with PMWS throughout the world (Segalés 2015).

PCV2 is a very small, non-enveloped DNA virus that belongs to the genus *Circovirus* and family *Circoviridae*. The genome is 1.76 kb, circularly arranged, negative sense, single-stranded (Opriessnig and Langohr 2013). This genome is composed of 11 open reading frames (ORFs) (Hamel 1998) and genomic analysis has done by phylogenetic analysis of the replicase gene (ORF 1) and capsid gene (ORF 2).

Results of the phylogenetic analysis proved PCV1 and PCV2 may have an unidentified common ancestor because they share about 80% nucleotide sequence identity (Hamel 1998; Olvera 2007). Furthermore, based on the nucleotide sequence identity two major genotypes named PCV2a and PCV2b were identified (Olvera 2007). From 1996 to 2000 PCV2a predominated but PCV2b became the predominant subtype in North American and European swine (Cortey 2011). In 2008, the third genotype was isolated in Denmark and described as PCV2c (Dupont 2008, Segalés 2008), and PCV2d and PCV2e have been identified in China (Wang 2009). However, the virulence of these different genotypes has not been evaluated (Harding 2010, Opriessnig 2006).

1.5.2 PCV2 pathogenesis

PCV2 can be transmitted through body secretions, saliva, urine, feces, milk, and semen but also as an airborne infection and by consumption of infected raw meat (Krakowka 2000, Larochelle 2000, Opriessnig 2009, Park 2009, Segalés 2005).

The host cell types associated with the pathogenesis of PCV2 are still not fully identified (Oppriessing 2007). However, severely affected pigs are suggestive of immunosuppression and the host may become more susceptible to the secondary bacterial infections (Segalés 2004).

1.5.3 PCV2 clinical disease

PCV2 infections are common throughout North America due to the ubiquitous nature of the

virus. Respiratory PCV2 infections are usually involved in PRDC (Kim 2003). The respiratory manifestations of PCV2 infection include respiratory distress, sneezing, coughing, nasal discharges and poor growth rate (Opriessnig 2007).

As a multifactorial disease, both infectious and non-infectious factors contribute to PCVAD (Grau-Roma 2011). Co-infections with pathogens such as porcine parvovirus and PRRSV have been associated with PMWS, and non-infectious factors such as stress, poor management practices, nutritional status and vaccination status are also important (Harding 1997, Rose 2003). The main clinical signs of PMWS are considerable weight loss and generalized lymphadenopathy. Other clinical signs include: dyspnea, pallor, jaundice, diarrhea, coughing, pyrexia and central nervous system disorders (Harding 2004).

PCV2-associated enteritis is common among grower-finisher pigs and it results in dark-colored diarrhea (Opriessnig 2007). This clinical outcome is often similar to *Lawsonia intracellularis* associated enteritis and enteric salmonellosis (Opriessnig 2011). Porcine dermatitis and nephropathy syndrome (PDNS) is characterized by acute onset of raised coalescing red to purple skin lesions in skin that can have crusts with black centers. The skin on the rear legs is most frequently affected (Opriessnig 2012).

PCV2 associated reproductive failure is seen in pregnant females. It is characterized by pre-weaning mortalities, late-term abortions, stillbirths, fetal mummifications and macerations at different stages (O'Connor 2001). Due to its rare occurrence, it is not considered to have a large economic impact (Opriessnig 2012). However, it can have severe economic consequences for individual producers if affected.

1.5.4 PCV2 pathologic lesions

PCV2-associated gross and histological lesions can be seen in a number of organs and tissues.

Gross lesions of PCV2 are easily observed in lymphoid tissues such as the spleen, lymph nodes, Payer's patches and tonsils (Opriessnig 2012). For PMWS, it can be lymphadenopathy or lymphoid atrophy of lymphoid tissue according to the stage of PMWS. Histologically, lymphocyte depletion and infiltration of large histiocytic cells and multinucleated giant cells are the characteristic lesions of PMWS-affected pigs (Rosell 1999). Additionally, sharply demarcated, spherical, basophilic cytoplasmic inclusion bodies within macrophages can see in affected lymphoid tissues (Rosell 1999).

Since the respiratory manifestation results in interstitial pneumonia, the affected lungs can become overinflated and rubbery in consistency, and are described as failing to collapse when the thorax is opened during the necropsy. Histological lesions of interstitial pneumonia with increased numbers of macrophages in the septae and alveolar spaces can be accompanied by peribronchial fibrosis and fibrinous bronchiolitis in advanced cases (Segalés 2004).

Digestive system associated lesions can be seen in the ileum, colon, and liver. Grossly, thick intestinal mucosa, gastric ulceration and enlarge mesenteric lymph nodes are common. Microscopically, granulomatous enteritis characterized by high numbers of macrophages and multinucleated giant cells can be seen in the small and large intestinal mucosa and sub-mucosa. Moreover, inflammation and necrosis can be seen in the liver of affected animal (Opriessnig 2012).

Histological lesions in the reproductive tracts of PCV2 infected sows are seldom seen. However, microscopic lesions of myocarditis are often seen in stillborn and neonatal pigs (Opriessnig 2012).

In PDNS, often both skin and renal lesions occur in the same animal. Bilateral renomegally with petechial hemorrhages and waxy appearance of kidneys are the characteristic gross lesions, while

fibrinonecrotizing glomerulitis and systemic vasculitis are observed microscopically (Segalés 1998, Opriessnig 2007). In the skin, grossly visible crusted lesions have microscopic features of necrotizing dermatitis.

1.5.5 PCV2 diagnostics

Diagnosis of PCV2 is based on the presence of characteristic microscopic lesions of the affected organs and the presence of PCV2 antibodies or antigens or nucleic acids in those lesions (Oppriessnig 2012). PCV2 assays include: *in situ* hybridization (ISH), IHC, ELISA, IFA, SN, virus isolation, electron microscopy (EM), PCR, restriction fragment length polymorphism (RFLP) and gene sequencing (Oppriessnig 2007). Among these techniques, IHC and ISH are often used as gold standards of PCV2 diagnosis. Though all these conventional techniques are usable, PCR has been increasing its popularity (Opriessnig 2012).

ELISA, IFA, and SN are useful to diagnose PCV2 antibodies. PCR and ISH techniques are important to identify PCV2 nucleic acids on a herd basis. On the other hand, IHC, EM, and IFA techniques are used to detect PCV2 antigens in tissue sections on an individual basis. Although a less commonly used technique, virus isolation of PCV2 virus is done on PK–15 cells (Opriessnig 2007).

1.5.6 PCV2 prevention and control

To minimize the economic loss, control and prevention of PCV2 in the form of management practices and vaccination policies is essential. Management practices focus on eliminating predisposing factors and co-infections while stimulating herd immunity and reduction of stress (Opriessnig 2007). Vaccines are useful when the condition is difficult to control by preventive measures. Currently, commercially produced inactivated virus vaccines with an oil-in-water adjuvant are highly used for grower-finisher pigs (Kekarainen 2010) and autogenous vaccines

are less commonly used. Additionally, anti-inflammatory drugs use as complementary treatments (Opriessnig 2007).

1.6 Oral fluids

Oral fluid is a combination of saliva secreted from the parotid, mandibular and sublingual salivary glands, and the transudate from oral mucosal circulation (Prickett 2010b). Oral fluids contain water, proteins, electrolytes, small organic molecules, antibodies, inflammatory cells, epithelial cells, food debris, bronchial-nasal secretions and microbiota (Detmer 2011, Gutierrez 2012). Functionally, OF plays a critical role in food ingestion, digestion, and natural defense mechanisms. Furthermore, OF contains IgA, IgM and IgG antibodies derived from serum (Prickett 2010b). However, the immunoglobulin composition of serum and OF is different. In fact, serum contains more IgM initially and later IgG while IgA is predominant in OF (Liew 1984).

1.6.1 History of oral fluid usage

The first reported use of OF in human medicine in 1909 points to a long history as a disease diagnostic sample (Gutierrez 2012). Since then, human OF has been used for routine testing for several tests including human immunodeficiency virus (HIV), drugs, hormones and antibodies (Detmer 2011, Kittawornrat 2012). However, the use of swine oral fluids as a diagnostic sample was first reported for PRRSV Iowa in 2008 (Opriessnig 2013), and it has since become a routine sample in the Midwestern United States. Swine oral fluid is currently being used to detect PCV2, PRRSV (Gutierrez 2012, Prickett 2010b) and IAV-S (Detmer 2011). Antibodies in swine oral fluid were first identified for classical swine fever in 1976 (Corthier 1976). Influenza A virus nucleoprotein antibodies in swine were first detected in 2013 and 2014 (Panyasing 2013, Panyasing 2014). Additionally, PRRSV-specific antibodies (Decorte 2014), anti-*Erysipelothrix*

rhusiopathiae antibodies (Gimenez-Lirola 2013), antibodies against African swine fever (Mur 2013), foot-and-mouth disease virus (Vosloo 2013) were detected in swine OF.

The popularity of OF is on the rise because it is an easy, reliable, safe, and non-invasive method that requires no special equipment or personnel (Detmer 2011, Escribano 2012, Gutierrez 2012, Prickett 2010b, Romagosa 2012, Seddon 2012). Additionally, it can be cost saving because it is a pooled sample representing 5-7 pigs that can increase the number of pigs being tested in a barn without increasing the number of tests being conducted.

1.6.2 Oral fluid sampling

OF represents a pooled sample from a group of pigs within the same pen, but it can be used for individual sampling according to the purpose. OF is collected using cotton ropes that are suspended at shoulder height for the pigs. The normal behavior of chewing on things is useful for this sampling process because the pigs are curious and often enjoy chewing on the ropes. For some pigs, such as recently weaned pigs, acclimatization few days before OF collection may be needed (Detmer 2011). Usually, 20 to 30 minutes is a sufficient exposure time to chew the ropes and OF extraction using mechanical compression within a plastic bag helps with sample collection (Prickett 2008).

1.6.3 Oral fluid diagnostics

OF has limitations as a diagnostic sample. Detmer 2011 described mucin and glycoprotein in human oral fluid that may act as inhibitors and neutralizers against IAV strains. Furthermore, the environmental temperature may affect the viability of infectious agent in the sample. However, the effect of PCR inhibitors (Chittick 2011) and viability of infectious agent after sampling (Detmer 2011) in swine saliva is unknown since there have been limited investigations into these factors. Moreover, the composition of oral fluid is not identical to serum. Although it contains

IgA, IgM and IgG antibodies, their concentrations are different in serum and OF. For instance, the predominant antibody in OF is IgA (Prickett 2010b) compared to IgG in serum. Additionally, the sensitivity and specificity of genomic material detection of swine OF are less compared to a gold standard. Therefore, providing the optimal conditions for the OF samples is essential during the transportation. In fact, it is crucial to maintain the sample on ice and transport to the laboratory as soon as possible. OF stabilizers have also been investigated to improve results (Decorte 2013).

Despite these limitations, the ease of use and accessibility of OF make it a good sample for surveillance when used with highly sensitive PCR assays for PRRSV, IAV-S, and PCV2.

1.7 HYPOTHESES AND OBJECTIVES

This research aims to determine the frequency of detection of the PRDC pathogens PRRSV, IAV-S and PCV2 on a select number of farms in Saskatchewan, Canada.

To accomplish this, the diagnostic PCR protocols used by PDS were validated for use with oral fluids. This included the evaluation of the efficiency ratio and the limit of detection based on the titer of $TCID_{50}$ for each of the viruses (Chapter 2). After that, PRDC surveillance was performed over two winters, and the frequency of detection of PRDC viruses in SK was determined (Chapter 3).

1.7.1 Null hypothesis

There was no detection of PRDC pathogens in pig oral fluids from selected swine barns in Saskatchewan, and there were no monthly trends of each of the three PRDC viruses over a two-year period.

1.7.2 Objectives

Even though PRDC is worldwide, very little information is published about the prevalence of these pathogens in Saskatchewan. It is essential to characterize both the pathogens and their prevalence to understand their epidemiology and identify proper control measures. Additionally, examining the monthly trends of each of the three PRDC viruses is important to have an idea about the seasonal pattern of the condition. Moreover, the purpose of this study is to assess the value of using oral fluids compared to the already ongoing on-farm routine disease surveillance being performed using the "gold-standard" samples of nasal swabs for IAV-S. This comparison of OF with the gold standard for IAV-S infections in swine populations is important for swine producers and workers because of the zoonotic potential.

1) To determine the detection frequency of the three PRDC viruses on selected farms within SK.
2) To examine the monthly trends of each of the three PRDC viruses based on the detection of viral genome in oral fluid samples.
3) To evaluates the positive frequency of OF and NSSW by sample, barn and month for IAV-S detection.
4) To compare the genetic types of IAV-S strains detected in SK by OF and NSSW.

2 VALIDATION OF DIAGNOSTIC RT-PCR PROTOCOLS FOR PORCINE RESPIRATORY DISEASE COMPLEX (PRDC) CAUSING PATHOGENS IN ORAL FLUIDS.

In Chapter 2, the assays used for the main study are validated. Validation is an important step for the main research project in Chapter 3, which uses these assays to test field samples.

Author contributions: S. Kulanayake and S.E. Detmer, conducted the sample collection and laboratory testing for this study. S. Kulanayake, S.E. Detmer, and T. Dumonceaux contributed to conception and design of the study, acquisition, analysis and interpretation of the data, and writing of the manuscript. J.C.S. Harding critically reviewed the manuscript.

2.1 Abstract

Conducting an assay validation is important to predict the diagnostic performance of a diagnostic assay. Therefore, validation of real-time PCR procedures is crucial for accurately diagnosing diseases such as PRDC pathogens: porcine reproductive and respiratory disease syndrome virus (PRRSV), influenza A (IAV-S) virus and porcine circovirus type 2 (PCV2) in oral fluids. During the validation process, eight ten-fold dilutions (10^0 to 10^{-7}) were made of each virus in sterile deionized water and negative oral fluids. RNA and DNA were extracted two ways using a machine for automated extraction and a column-based kit for manual extraction. Real-time, reverse transcription PCR (RRT-PCR) for PRRSV and IAV-S were performed, and an in-house SYBR Green assay was used for the PCV2 assay. The amplification efficiencies for manual extraction were determined to be: 94.92% (R^2 = 0.99096) and 71.67% (R^2 = 0.9887) for PRRSV OF dilutions and water dilutions respectively. However, 100.42% (R^2 = 0.99971) and 101.74% (R^2 = 0.97125) amplification efficiencies were obtained by machine extraction for PRRSV OF dilutions and water dilutions respectively. For IAV-S manual extraction, 93.2% (R^2 =0.99638) and 110% (R^2 =0.9924) amplification efficiencies were obtained for OF dilutions and water dilutions respectively while 76.77% (R^2 = 0.98534) and 85.7% (R^2 =0.9917) efficiencies were obtained by machine extraction. Moreover, 74.61% (R^2 = 0.9959) and 57% (R^2 = 0.97286) RT-PCR efficiencies were determined for manual extraction for PCV-2 OF dilutions and water dilutions. RT-PCR efficiencies of machine extraction for PCV2 OF dilutions and water dilutions were 78.9% (R^2 = 0.9778) and 99.49% (R^2 = 0.99534) respectively. The lowest detectable amount or analytical sensitivity of PRRSV was 500 $TCID_{50}$/ml by both machine extraction and manual extraction while the lowest detectable amount of IAV-S was 20000 $TCID_{50}$/ml by machine extraction and 2000 $TCID_{50}$/ml by manual extraction. The lowest detectable amount of

PCV2a was 4000 $TCID_{50}$/ml by both machine extraction and manual extraction. Further validation will be required to test optimization using this baseline results in the future.

2.2 Introduction

It is expected that a diagnostic technique gives the true status of a sample in infectious disease diagnosis. Validation of a laboratory technique is a crucial factor for assuring that test results reflect the true status of the samples (Burkardt 2000). Moreover, validation is the evaluation of a diagnostic assay for the purpose of determining how suitable for the purpose it is used. Therefore, conducting an assay validation is important to predict the performance of a diagnostic assay (OIE 2013).

Test validation is important for producing good quality data. The data obtained from a properly validated test has important qualities, such as accuracy, precision, the range of reportable results, reference interval, analytical sensitivity and analytical specificity (Burd 2010). When validating a PCR assay, the efficiency of the assay should also be determined (Pfaffl 2001). Therefore, validation of the assay is crucial for infectious disease diagnosis.

In order to examine porcine respiratory disease complex (PRDC) in Saskatchewan (SK), three viruses were selected for validation: porcine reproductive and respiratory disease syndrome virus (PRRSV), influenza A virus in swine (IAV-S) and porcine circovirus type 2 (PCV2). The objective of the present study is to validate these three real-time PCR assays for oral fluid samples.

2.3 Materials and Methods

2.3.1 Oral fluid collection

Swine oral fluids (OF) were selected as the sample of choice for detecting PRDC pathogens. Over 100 ml of OF was collected from pigs known to be negative for PRRSV, IAV-S and PCV2 under an animal care and use protocol (ACUP#20130116) approved by the University of Saskatchewan's Animal Research Ethics Board, and adhered to the Canadian Council on Animal

Care guidelines for humane animal use. The OF was centrifuged at 9000×*g* for 10 minutes to remove particulate debris and the supernatant was transferred to 15 ml centrifuge tubes in 10 ml aliquots and frozen at -80°C until assayed. The disease status of the pigs was verified by serum ELISA assays at Prairie Diagnostic Services, Inc. (PDS). The OF was verified to be negative for PRRSV, IAV-S and PCV2 by real-time PCR using validated assays at the University of Minnesota Veterinary Diagnostic Laboratory (UMVDL).

2.3.2 Viruses

To determine the real-time PCR efficiencies, pure virus isolates were obtained. The PRRSV used was NVSL 97-7895 (PRRS A) at 5.0 x 10^7 TCID50/ml (Ladinig 2015), influenza A/swine/Texas/4199-2/1998 H3N2 (TX98) at $10^{6.3}$ TCID50/ml (Detmer 2013b), and OSUp4 (PCV2a) at 4 x 10^8 TCID50/ml (Harding 2010). Eight ten-fold dilutions (10^0 to 10^{-7}) were made of each virus in sterile deionized water, and these were spiked into 1 ml each of water and negative oral fluids to make dilutions of 10^{-1} to 10^{-7}.

2.3.3 RNA and DNA Extraction

RNA and DNA were extracted two ways using a machine for automated extraction and a column-based kit for manual extraction as described below.

2.3.3.1 Machine extraction

For the RNA extraction, 100 µl of sample (virus in water or virus in OF) was used and the same RNA extract was used for both the PRRSV and IAV-S assays. The internal control for IAV-S, Armored RNA® Enterovirus, (Ambion Diagnostics Inc., Austin, Texas USA; Asuragen Catalog# 42050) was added to the lysis buffer. The BioSprint 96 One-For-All Vet Kit (Qiagen Inc., Valencia, California, USA; Catalog# 947057) was used on the BioSprint 96 workstation, according to manufacturer's instructions. The viral RNA was eluted into 70 µl of RNase and

DNase free water.

For the DNA extraction, 200 µl of sample was used, and 75 µl of RNase and DNase free water was used to elute. Phosphate buffered saline (PBS) was used as the negative extraction control (NEC) and an NEC was prepared with each extraction. The DNA and RNA were immediately stored at -70°C after extraction.

2.3.3.2 Manual extraction

For the RNA extraction, 500 µl of sample (virus in water or virus in OF) was used, and the same RNA extract was used for both the PRRSV and IAV-S assays. The RNA was extracted using the RNeasy Mini kit (Qiagen; Catalog# 74106) according to manufacturer's instructions. The internal control for IAV-S, Armored RNA® Enterovirus, was added to the lysis buffer and 50 µl of RNase and DNase free water was used to elute the RNA.

For the DNA extraction, 200 µl of sample was used with the DNeasy Blood and Tissue kit (Qiagen; Catalog# 69506), and 100 µl of RNase and DNase free water was used to elute. Phosphate buffered saline (PBS) was used as the negative extraction control (NEC) and an NEC was prepared with each extraction. The DNA and RNA were immediately stored at -70°C after extraction.

2.3.4 PCR Assays

Primer sets for PRRSV, IAV-S and PCV2, were selected for highly conserved genes for each pathogen. The PRRSV assay is a proprietary kit, the IAV-S assay is a CFIA protocol similar to a published protocol (Sponseller 2010), and the PCV2 primers are published (Table 2.1). The master mixtures for PRRSV, IAV-S and PCV2, were prepared in clean, 1.5-ml nuclease-free microcentrifuge tubes and reaction tubes were placed in a cooling block while transferring the master mix. Aerosol-resistant pipette tips were used throughout the procedure. Positive controls

for the PCR tests were provided by PDS, along with the CFIA IAV-S and PCV2 primer probe sequences (Table 2.1). Internal positive control, positive test control, negative test control and extraction blank were used with each testing batch.

2.3.4.1 PRRSV

Real-time, reverse transcription PCR (RRT-PCR) for PRRSV was performed with commercially available reagent sets (Tetracore Inc., Rockville Maryland USA; Catalog# TC-9060-096) for the specific detection and differentiation of North American and/or European PRRSV RNAs by reverse transcribing into complementary DNA (cDNA). The amplification was done in a single-tube, one-step differential RRT-PCR reaction. The master mix contained primers and probes targeting NA and EU PRRS viral RNA with Inhibition Control (IC). Master mix component volumes per well consisted of 17.25 µl (included a buffer, primers, and probes) and 0.75 µl of enzyme blend. Ultimately, 18 µl of master mix was combined with 7 µl of PRRSV RNA extract into RRT-PCR tube strips. The strips were covered by Masterclear™ Cap Strips and the strips were centrifuged briefly before being loaded onto the thermal cycler. RRT-PCR was performed using a Mx3000P Stratagene platform (Stratagene, La Jolla, CA) using the following thermal cycling conditions: one cycle at 48°C for 15 min, one cycle at 95°C for 2 min, 45 cycles of: 95°C for 5 seconds, 60°C for 40 seconds. A cycle threshold (C_t) of 38 was considered cut off level for PRRSV according to the manufacturer's instructions.

2.3.4.2 IAV-S

RRT-PCR for IAV-S was performed using CFIA developed "Influenza A 2009 matrix" procedure with the commercially available kit (AgPath-ID™ One-Step RT-PCR kit, Applied Biosystems, Foster City, CA, USA). Master mix component volumes per well consisted of 12.5 µl of 2X RT-PCR buffer, 0.4 µM INF- A 2009 matrix For primer, 0.8 µM INF- A 2009 matrix

Rev primer, 0.2 µM Entero 31- For primer, 0.2 µM Entero 31- Rev primer, 0.5 µl of 6 µM AIV Matrix + 64 probe, 0.25 ul of 6 µM Entero 31- probe, 1.0 µl of 25X enzyme mix and 0.75 µl of RNase- Free water. Finally, 17 µl of master mix was combined with 8 µl of IAV-S RNA extract into RT-PCR tube strips. RT-PCR was performed by Mx3000P Stratagene platform using the following thermal cycling conditions: one cycle at 45°C for 10 min, one cycle at 95°C for 10 min, 40 cycles of 94°C for 5 seconds, 60°C for 60 seconds. Detection of amplified target was accomplished by hydrolysis probe chemistry, and the internal control was used to detect nucleic acid extraction failures. A cycle threshold (C_t) of 35 was considered cut off level for IAV-S according to the manufacturer's instructions.

2.3.4.3 PCV2

An in-house SYBR Green assay was used for the PCV2 assay (McIntosh 2009). This RT-PCR assay for PCV2 was performed using commercially available reagent sets (SYBR GreenER™ qPCR SuperMix Universal, Life Technologies™). Each quantitative real-time PCR was performed using 12.5 µl of SYBR Green Mastermix, 0.15 µM each primer (PCV2-83F and PCV2-83R), 30 nM reference dye, 5.66 µl of sterile ultra- pure water and 3 µl of template. Real-time PCR was performed using a Mx3000P Stratagene platform using the following thermal cycling conditions: 10 min at 95°C, followed by 45 cycles of 30 s at 95°C, 1 min at 59°C, and 30 seconds at 72°C. A cycle threshold (C_t) of 35 was considered cut off level for PCV2 (Harding 2010).

2.3.5 Standard curves

Amplification efficiency of PCR is the rate at which amplicon is generated during the geometric phase. It commonly expressed as a percentage. If the amount of PCR product is doubled during the geometric phase, the PCR efficiency reaches 100% (Applied biosystems guide). Furthermore,

the efficiency measures the overall performance of the real-time PCR assay. Real-time PCR assays were carried out for each dilution series and the standard curves were generated. Standard curves were graphically represented as a semi-log regression line plot of Ct value vs. log of input nucleic acid. Data points from highly concentrated and highly diluted samples were eliminated to obtain linear curves. Then, the slopes of the linear standard curves and correlation coefficient (R^2) were calculated. The slope of a standard curve is used to estimate the PCR amplification efficiency using $E = 10^{(-1/slope)}$ (Pfaffl 2001).

2.3.6 Sensitivities and specificities

To obtain the lowest detectable amount of IAV-S and PRRSV in an oral fluid sample based on the titer of the pure virus, the following experiments were conducted. Eight ten-fold serial dilutions were prepared using aliquots of virus-negative oral fluids, which spiked with strain influenza A/swine/Texas/4199-2/1998 H3N2 (TX98) (initial titer = 2×10^6 $TCID_{50}$/ml) and NVSL 97-7895 (PRRS) (initial titer = 5×10^7 $TCID_{50}$/ml), separately. To evaluate the analytical specificity of the IAV-S real-time RT-PCR assay, different IAV-S virus strains were used (IL08, TX98).

2.4 Results

2.4.1 PRRSV

Using the slope of real-time PCR amplification plots as described; the amplification efficiencies for manual extraction by column method were determined to be: 94.92% (R^2 = 0.99096) and 71.67% (R^2 = 0.9887) (Fig 2.1) for PRRSV OF dilutions and water dilutions respectively. However, 100.42% (R^2 = 0.99971) and 101.74% (R^2 = 0.97125) (Fig 2.2) amplification efficiencies were obtained by machine extraction for PRRSV OF dilutions and water dilutions respectively (Table 2.2).

2.4.2 IAV-S

For IAV-S manual extraction, 93.2% (R^2 = 0.99638) and 110% (R^2 = 0.9924) (Fig 2.3) amplification efficiencies were obtained for OF dilutions and water dilutions respectively while 76.77% (R^2 = 0.98534) and 85.7% (R^2 = 0.9917) (Fig 2.4) efficiencies were obtained by machine extraction (Table 2.2).

2.4.3 PCV2

According to the calculations, 74.61% (R^2 = 0.9959) and 57% (R^2 = 0.97286) (Fig 2.5) RT-PCR efficiencies were determined for manual extraction for PCV-2 OF dilutions and water dilutions. RT-PCR efficiencies of machine extraction for PCV2 OF dilutions and water dilutions were 78.9% (R^2 = 0.9778) and 99.49% (R^2 = 0.99534) (Fig 2.6) (Table 2.2) respectively.

2.4.4 Sensitivities and specificities

The lowest detectable amount or analytical sensitivity of PRRSV was 500 $TCID_{50}$/ml by both machine extraction and manual extraction while the lowest detectable amount of IAV-S was 20000 $TCID_{50}$/ml by machine extraction and 2000 $TCID_{50}$/ml by manual extraction. The lowest detectable amount of PCV2a was 4000 $TCID_{50}$/ml by both machine extraction and manual extraction.

The analytical specificity of real-time RT-PCR assay was detected by different IAV-S virus strains (IL08, TX98) and only TX98 was detected by the test.

2.5 Discussion

In the validation procedure, real-time PCR efficiencies and correlation coefficients (R^2) were obtained, which were two of the most important determinants of a validation procedure. Some of the efficiencies and correlation coefficients (R^2) were not in the optimal range, and this result was somewhat expected based on published results of OF assays.

The standard curve slope of –3.32 indicates the ideal situation with 100% PCR efficiency (Applied Biosystems guide). In fact, if a 10-fold increase in PCR amplicons occurs every 3.32 cycles during the exponential phase of amplification ($\log_2 10 = 3.3219$) a PCR will obtain 100% efficiency. Normally, slopes of the regression curve between -3.1 and -3.6 are acceptable, and those values indicate efficiency between 90% and 110% (Raymaekers 2009).

However, in our validation assay, we obtained slopes more negative than –3.32 and more positive than –3.32, as well. According to Applied Biosystems guide slopes more negative than –3.32 indicate efficiency less than 100%. Some of the possible reasons for reduced efficiency include poor primer design, suboptimal reaction conditions, and pipetting errors. The slopes more positive than –3.32 indicate efficiency more than 100%. This could be due to poor sample quality, PCR inhibition or nonspecific amplification, including primer-dimer formation.

The linearity of the standard curve is another most important determinant of a validation process, and it is measured by the correlation coefficient (R^2) of the standard curve (Broeders 2014). R^2 is a measure of the strength of relationship (Raymaekers 2009), and +1 indicates the perfect correlation (Burd 2010).

In real world situation, PCR efficiencies and correlation coefficients (R^2) can be out of the optimal range for several reasons, which reduce the diagnostic value of real-time PCR results. PCR inhibitors, PCR enhancers, template degradation, the concentration of the template, the size of the amplicon, secondary structure, and primer quality like any number of determinants can affect the efficiency of the PCR (Raymaekers 2009).

The quality of the template is one of the most important determinants, and it directly affects the reproducibility as well (Bustin 2004). Therefore, it is essential to provide the proper environment while collecting, transporting, processing and storing the sample to prevent template degradation,

as RNA is delicate compared to DNA (Bustin 2004). Moreover, to have reliable results, the template should be pure and free of nucleases (Bustin 2004).

The second most important determinant of real-time PCR result is PCR inhibitors in the sample. Swine OF is a possible source of PCR inhibitors as it is a mixture of numerous components like food particles, feces, microbes and substances from the environment. According to Rossen et al. (1992), PCR inhibitors are present in feed and swine saliva may contain a variable amount of food particles. Furthermore, Prickett et al. (2010a) discovered that a high number of bacteria containing (flora-dense) swine oral fluid samples act as inhibitors. If PCR inhibitors are suspected in the sample, purification of the template or finding a lower template concentration that cannot be affected by PCR inhibitors are possible solutions.

Normally inhibitors are highly concentrated in the least diluted samples with high template concentration, and they may give higher C_t values compared to the more diluted samples. Therefore, as the template is titrated, inhibitors are diluted as well and may give lower C_t values. Therefore, inconsistent C_t value differences between standard points can be expected with inhibition. On the other hand, consistent C_t difference between standard points with PCR efficiency higher than 100% may indicate a pipetting error.

Nonspecific amplification due to mispriming or primer–dimer formation is another disadvantage of PCR, which reduces its diagnostic value. Primer-dimer formation is comparatively common in SYBR assays, and it increases the necessity of doing post-PCR analysis such as sequencing or melt curve analysis. However, these artifacts do not generate a signal and are not detected in the hydrolysis assays due to the presence of the hydrolysis probe (Bustin 2004).

To increase the validity of real-time PCR assay, analysis of different controls is valuable. Obtaining no Ct values for negative extraction control in each run indicates no contamination

during the extraction or the amplification (Raymaekers 2009). Moreover, the use of a no template control (NTC) is important to detect reagent contamination or increased background signal. Positive amplification control (PAC) should be near the limit of detection of the assay but high enough to obtain reliable results. Adding an internal control (IC) is crucial in every assay to ensure the performance of the genome extraction and exclude false negative results from inhibition (Raymaekers 2009). Moreover, for more reliable results each dilution can be analyzed in triplicate (Raymaekers 2009).

The analytical sensitivity of real-time PCR is called the limit of detection (LOD), and it detects the lowest concentration of a given substance in a specimen with acceptable precision (Burd 2010, Broeders 2014). Analytical sensitivity of RT-PCR test experimentally can be determined by preparing a serial dilution of a template (Broeders 2014). In this assay, analytical sensitivity of both manual and machine extraction were same for PRRSV and PCV2. However, manual extraction was ten times more sensitive in IAV-S compared to machine extraction.

2.6 Conclusions

The analytical sensitivity, analytical specificity and efficiency of the real-time PCR obtained, were sufficient to conduct a diagnostic assay in a practical application. Also, baseline results of this assay will be a useful tool for future experiments.

Table 2.1 List of primer and probe sequences for the PCV2 assay (McIntosh 2009).

Primer/probe name	Sequence
PCV2	
PCV2- 83 F	5'-AAAAGCAAATGGGCTGCTAA-3'
PCV2- 83 R	5'- TGGTAACCATCCCACCACTT-3'

The kits used for PRRSV and IAV-S detection assays are proprietary.

Table 2.2 Slopes of the standard curves, correlation coefficients (R2) and amplification efficiencies of PRRSV, IAV-S, and PCV2 real-time PCR assays.

	PRRSV				IAV-S				PCV2			
	D.H_20 Dilution		OF Dilution		D.H_20 Dilution		OF Dilution		D.H_20 Dilution		OF Dilution	
	MaE	ME	MaE	ME	MaE	ME	MaE	ME	MaE	ME	MaE	ME
Slope	-3.46	-3.28	-4.26	-3.31	-2.91	-3.72	-3.5	- 4.82	-5.11	- 3.96	-4.13	-3.33
R^2	0.99	0.97	0.99	0.99	0.99	0.99	0.996	0.985	0.973	0.995	0.996	0.98
Efficiency	71.7 %	101.7 %	94.92 %	100.4 %	110 %	85.7%	93.2 %	76.77 %	57 %	99.49 %	74.6 %	78.9%

MaE: Manual extraction, ME: Machine extraction.

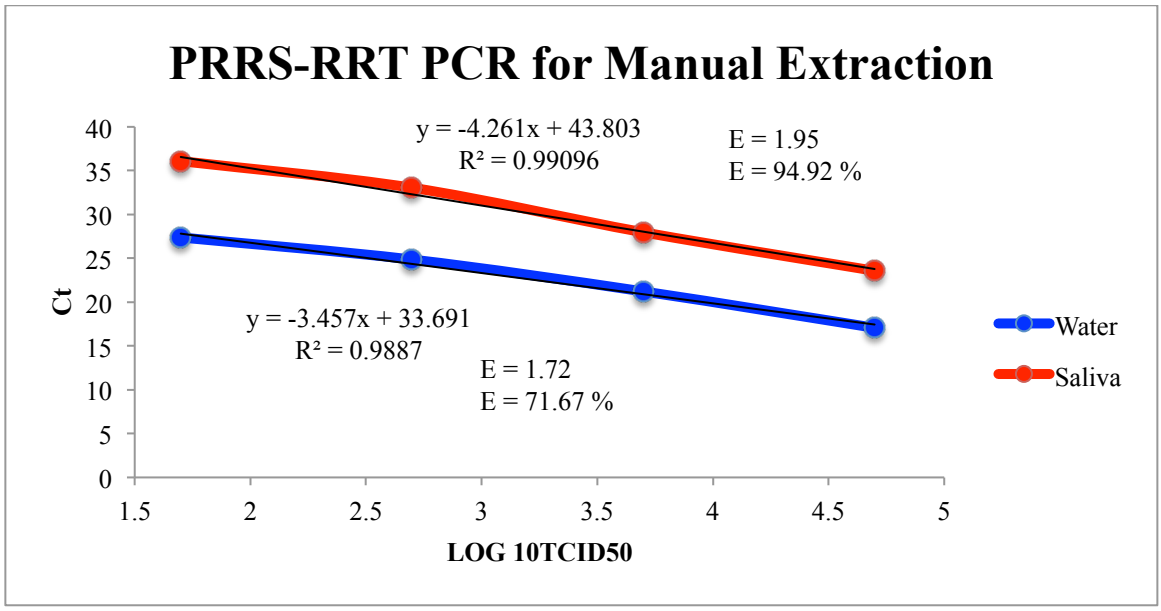

Figure 2.1 RRT-PCR Standard curves of PRRSV distilled water and OF dilutions by manual extraction.

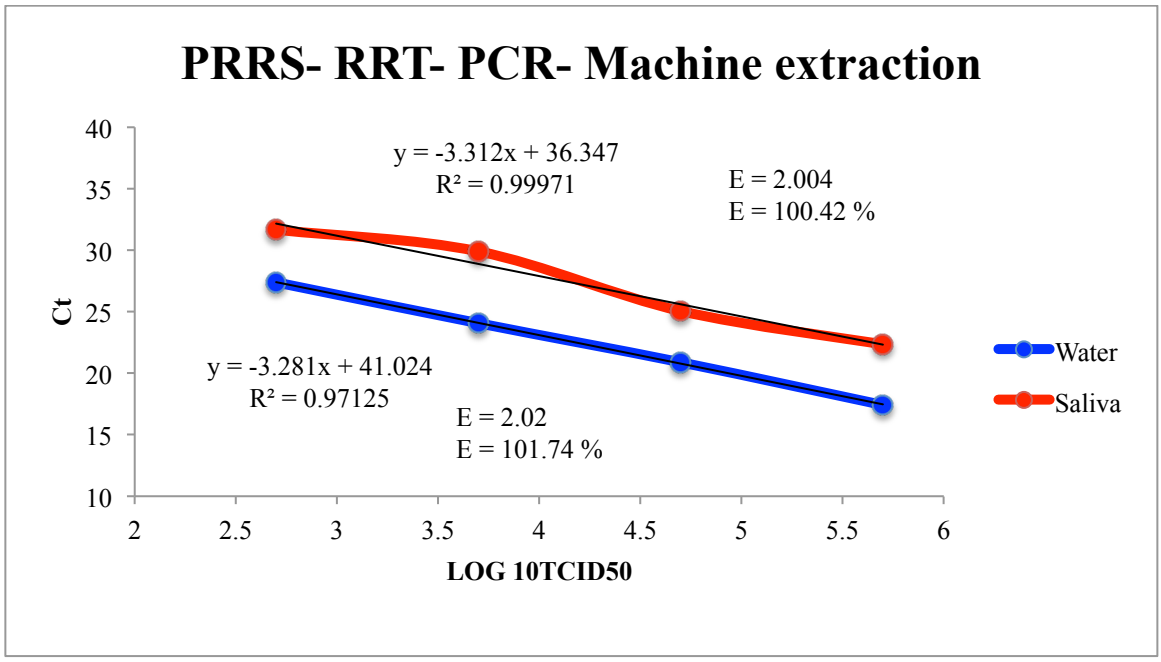

Figure 2.2 RRT-PCR Standard curves of PRRSV distilled water and OF dilutions by machine extraction.

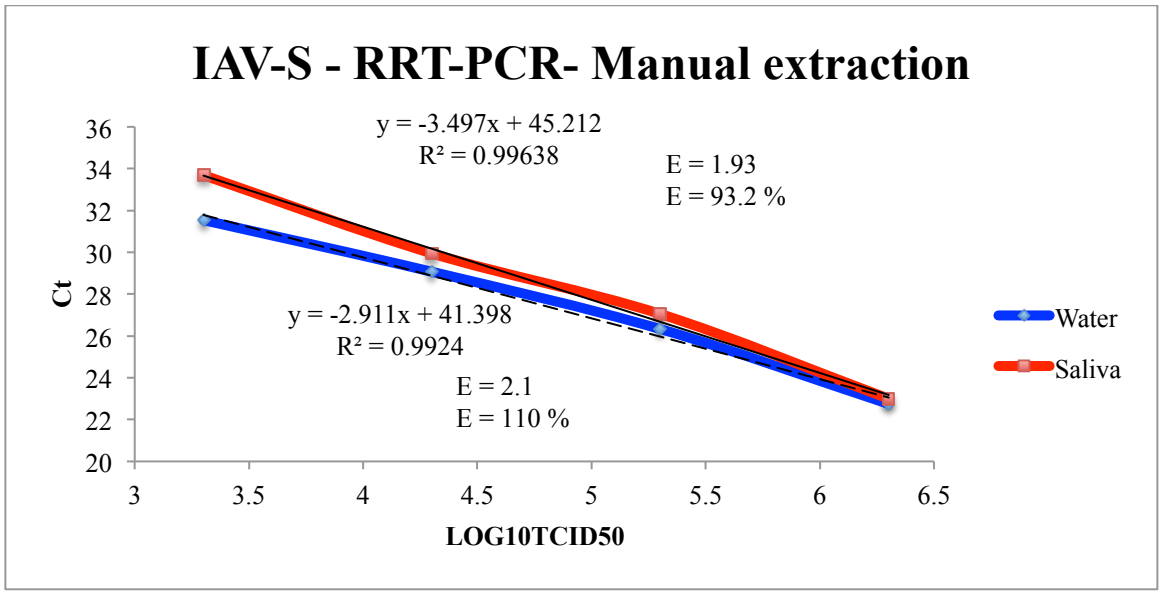

Figure 2.3 RRT-PCR Standard curves of IAV-S distilled water and OF dilutions by manual extraction.

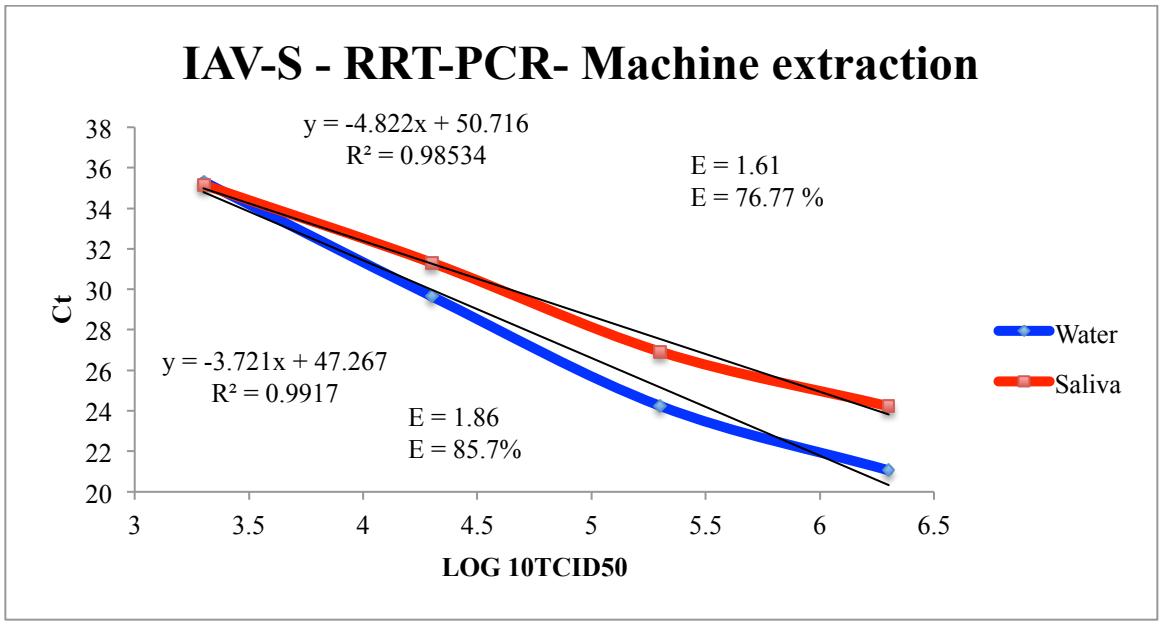

Figure 2.4 RRT-PCR Standard curves of IAV-S distilled water and OF dilutions by machine extraction.

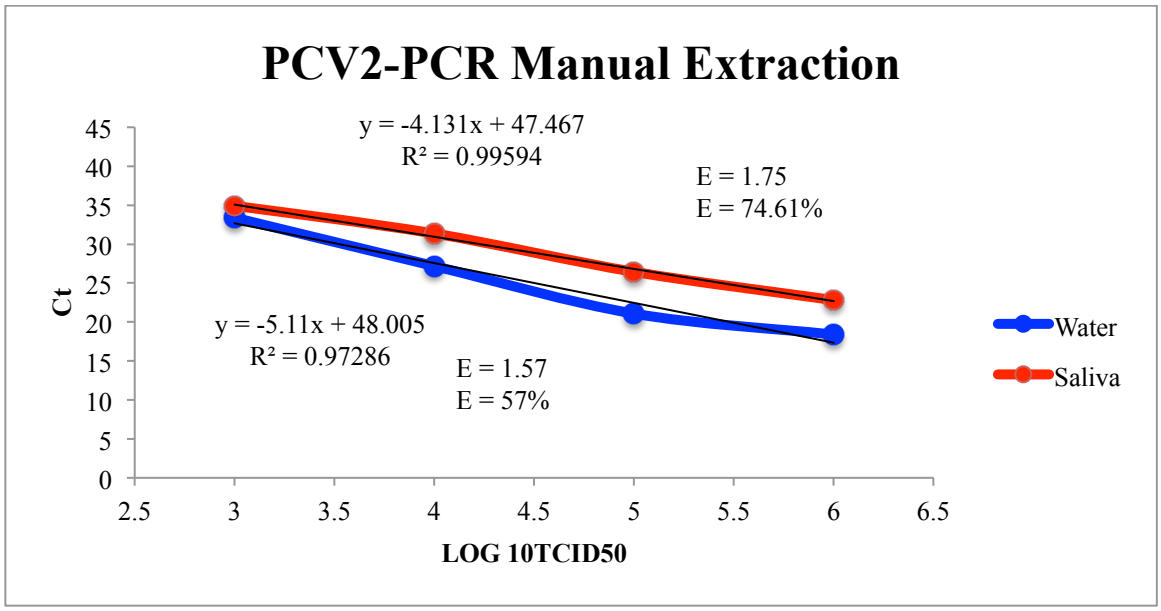

Figure 2.5 PCR Standard curves of PCV2 distilled water and OF dilutions by manual extraction.

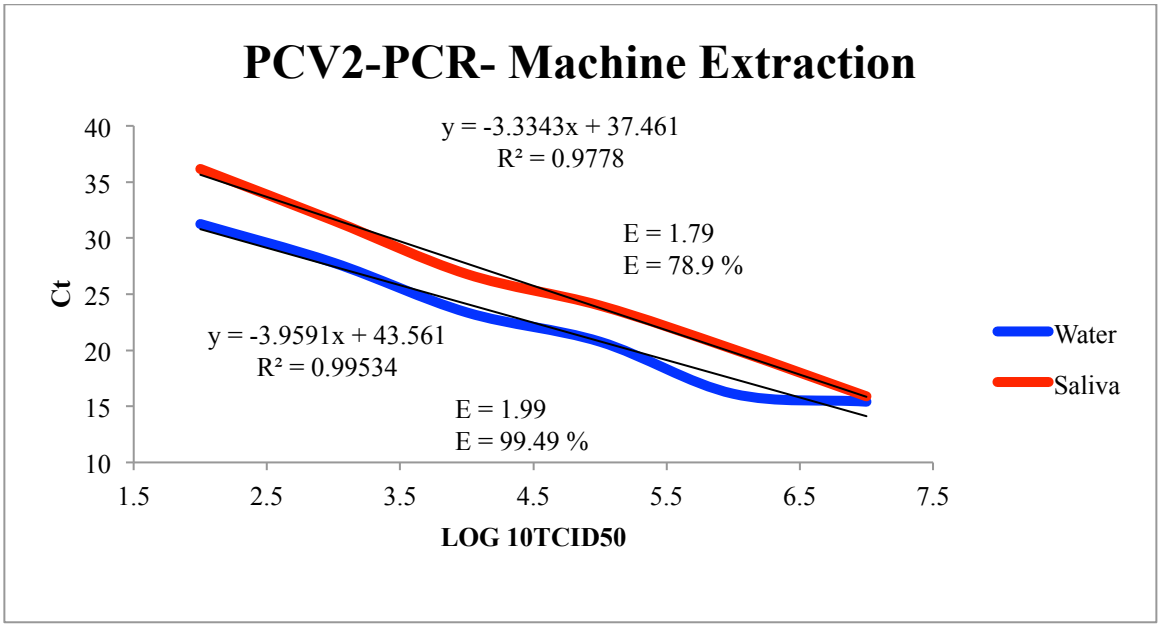

Figure 2.6 PCR Standard curves of PCV2 distilled water and OF dilutions by machine extraction.

3 PORCINE RESPIRATORY DISEASE COMPLEX (PRDC) DIAGNOSTIC TESTING USING ORAL FLUIDS.

In Chapter 3, twelve farms were enrolled in a field study over two years examining PRDC on Saskatchewan farms. Using the assays validated in Chapter 2, the field samples consisted of oral fluids from all farms over the two winters and nasal swabs from selected farms.

Author contributions: S. Kulanayake and S.E. Detmer, conducted the sample collection and laboratory testing for this study. S. Kulanayake, S.E. Detmer, T. Dumonceaux, and J.C.S. Harding contributed to conception and design of the study, acquisition, analysis and interpretation of the data, and writing of the manuscript.

3.1 Abstract

Porcine respiratory disease complex (PRDC) is an economically devastating disease complex among growing pigs worldwide. Three of the most common viral pathogens that play a role in PRDC are porcine reproductive and respiratory disease syndrome virus (PRRSV), influenza A (IAV-S) virus and porcine Circovirus type 2 (PCV2). Even though PRDC is worldwide, very little information is published about the prevalence of these pathogens in western Canada. Therefore, the purpose of this study is to assess the detection frequency of the three PRDC viruses on selected farms within Saskatchewan province using oral fluids (OF), to examine the monthly trends of each of the three respiratory viruses, to evaluates the positive frequency of OF and NSSW by sample, barn and month in grower pigs for IAV-S detection and to compare the genetic types of IAV-S strains detected in SK by OF and NSSW.

Six oral fluids (OF) samples were collected per month from each of ten and nine grower-finisher farms for five consecutive months over the winters of 2013-2014 and 2014-2015 respectively (509 samples total). Real-time PCR was used to detect viral nucleic acid in all OF samples. Barns were considered positive for a pathogen if at least one pen tested positive in a given month. During the first winter season, 257 samples were collected, and 46 (17.90%) were positive for PRRSV, 9 (3.50%) were positive for IAV-S, and 156 (60.70%) positive for PCV2. The barn-level prevalence of PRRSV, IAV-S and PCV2 were 50%, 30%, and 80% respectively. During the second winter season, 252 samples were collected, and 19 (7.54%) were positive for PRRSV, 16 (6.35%) were positive for IAV-S, and 76 (30.16%) positive for PCV2. The barn-level prevalence of PRRSV, IAV-S and PCV2 were 55.5%, 33.33%, and 88.89% respectively. More than one pathogen was found in six farms, and all three pathogens were found in one farm in both years. The barn-level prevalence in year 1 to year 2 was similar. However, the detection

frequency of three pathogens varied over the period of collection, and pathogens detected varied month to month.

The third objective evaluates the positive frequency of OF and NSSW by sample, barn and month in grower pigs for IAV-S detection was conducted using NSSW received with some of the oral fluid samples. These NSSW were tested for IAV-S by RRT-PCR and NSSW detection was more frequent than OF in this study. In conclusion, OF has high diagnostic specificity for IAV-S detection proving OF will be a reasonable sample of choice on farms with low IAV-S prevalence. Moreover, there was no effect of the season for sampling and currently circulating influenza viruses of these Saskatchewan swine farms were found to be alpha H1N1, 2009 pandemic H1N1, and Cluster IV H3N2.

3.2 Introduction

Porcine respiratory disease complex (PRDC) is one of the most economically significant swine diseases in growing pigs worldwide (Opriessnig 2011). It is predominantly seen in grower-finisher pigs between the ages of three and six months. The disease complex is multi-factorial, with both infectious and non-infectious factors contributing to disease (Opriessnig 2011). The clinical signs of PRDC are typical for respiratory diseases and include: anorexia, fever, coughing, dyspnea, lethargy, and decreased growth rate (Opriessnig 2011). The disease complex is characterized by 30-70% morbidity and 4-6% mortality (Opriessnig 2011)

The most common viruses that play a role in PRDC are porcine reproductive and respiratory disease syndrome virus (PRRSV), influenza A virus of swine (IAV-S) and porcine circovirus type 2 (PCV2) (Sibila 2009). The bacterial pathogen most often associated with PRDC is *Mycoplasma hyopneumoniae* (Mhyo). Other pathogens include pseudorabies virus (PRV), porcine respiratory corona virus (PRCV), *Bordetella bronchiseptica, Actinobacillus pleuropneumoniae, Pasteurella multocida, Haemophilus parasuis, Streptococcus suis, Actinobacillus suis, Salmonella choleraesuis, Arcanobacterium pyogenes* (Sibila 2009). Two or more these pathogens typically infect the pigs at the same time, making it polymicrobial in nature (Opriessnig 2011). Non-infectious factors include the environment, population size, management strategies and pig health (Opriessnig 2011).

Inhalation is considered the most common transmission route for these viruses within and between swine herds (Opriessnig 2011). PRRSV and PCV2 both replicate in macrophages (Chand 2012, Linhares 2012, Opriessnig 2011). PCV2 also replicates in bronchiolar epithelium and endothelial cells (Opriessnig 2011) while IAV-S replicates in epithelial cells in both upper and lower respiratory tract (Opriessnig 2011). These pathogens decrease the efficacy of the

natural defense mechanisms of the respiratory tract, including the mucociliary apparatus, and can cause systemic immunosuppression while inducing inflammation (Opriessnig 2011).

To detect the presence of these pathogens on a farm: antemortem samples include nasal swabs (NSSW) for IAV-S and serum for PCV2 and PRRSV. Postmortem samples include lung tissue for all three viruses but bronchial swabs, trachea and nasal turbinate can also be used for IAV-S. In this study, we used real-time PCR to detect viral nucleic acid in swine OF, which has become a popular alternative sample for these viruses in the midwestern USA. It is currently being used to detect PRRSV (Gutierrez 2012, Prickett 2008, Prickett 2010b) and IAV-S (Detmer 2011) RNA and their antibodies.

OF sampling is an easy, reliable, cost effective and non-invasive method (Detmer 2011, Escribano 2012, Gutierrez 2012, Prickett 2008, Romagosa 2012, Seddon 2012). However, the effect of PCR inhibitors in saliva (Chittick 2011) and viability of infectious agent after sampling (Detmer 2011) are not fully understood since there have been limited investigations into these factors.

This pilot project presented here aimed to familiarize Saskatchewan swine producers with the ease and efficiency of collecting these samples themselves, and also provided valuable experience to swine practitioners and diagnosticians that work with these producers as to the cost-effectiveness and efficiency of the new sampling methods.

3.2.1 Problem statement

Even though PRDC is worldwide, very little information is available in the literature about the prevalence of these pathogens in western Canada. Additionally, field strains of these pathogens in Saskatchewan (SK) are not available for sequencing and comparison to those present in other provinces. In order to perform surveillance and determine the true prevalence of PRDC viruses

in SK, baseline numbers are needed to determine an appropriate sampling regimen for an epidemiology study.

It is essential to characterize both the pathogens and their prevalence to understand their epidemiology and identify proper control measures. This project allowed us to identify field strains of PRDC pathogens that circulate among Saskatchewan pigs and work with producers to implement appropriate control measures.

3.2.2 Objectives

The main goal of this study is to examine PRDC pathogens on selected farms in Saskatchewan, Canada. There are three specific objectives that address this goal.

1) To determine the detection frequency of the three PRDC viruses on selected farms within SK.
2) To examine the monthly trends of each of the three PRDC viruses based on the detection of viral genome in oral fluid samples.
3) Evaluates the positive frequency of OF and NSSW by sample, barn and month for IAV-S detection.
4) To compare the genetic types of IAV-S strains detected in SK by OF and NSSW.

3.3 Materials and Methods

3.3.1 Experimental design

During the 2-year study, a total of 12 farms in Saskatchewan containing grower-finisher pigs were selected to participate in the study. Farm selection was based on producer and veterinarian willingness to participate, history of respiratory disease problems, the availability of routine herd test results for PRDC pathogens and the ability to collect and submit samples on a monthly basis.

Ten farms were enrolled in Year 1 and nine farms were enrolled in Year 2. Based on diagnostic records of the 12 farms total for PRRS, IAV-S, and PCV2, these pathogens have been detected on 6, 5, and 11 of these farms, respectively. Only three farms have had positive Mhyo serology, two of which did not submit enough samples to be included in the statistical analyses. Therefore, Mhyo was not examined in this study.

Most of the participating farms weaned at three weeks of age (20 to 24-days-old) and moved pigs from the nursery to a grower-finisher barn at 10 to 12-weeks-old. The age of the pigs sampled varied from 8 to 12-weeks-old. Sample collection was done once a month from November to March during 2013-2014 and 2014-2015 seasons, for a potential total of 600 OF samples.

Each month, the farm manager or veterinarian selected six pens of pigs those were close to 10-weeks-old to sample. The samples were taken from pens containing, at least, ten pigs and were evenly scattered throughout the room or rooms within the barn (Fig. 3.1). The oral fluid samples were collected from each pen using separate collection kits. For the monthly sampling, each barn was provided with six pre-labeled collection kits that contained: a re-sealable plastic bag, 50 ml pre-labeled centrifuge tube with screw cap, 3 feet long, 1.6 cm wide, 3-stranded cotton rope (www.webriggingsupply.com) and paperwork. The ropes were tied within the pen so that the end of the rope was at shoulder height on the pigs. The rope was removed after 20-30 minute exposure time and mechanically squeezed into the plastic bag. The corner of the bag was cut and the sample was poured into the centrifuge tube and as labeled. The tubes were placed in a shipping container with ice packs and shipped to the laboratory within 48 hours. The pen-based OF samples were centrifuged at 9000 $\times$ *g* for 10 minutes to remove particulate debris and the supernatant was transferred to two 4 ml cryovials and frozen at -80°C until assayed.

Qualified veterinarians were involved in the sample collection procedure, an online video (http://vetmed.iastate.edu/vdpam/disease-topics/oral-fluids) and a laminated poster of step-by-step procedure and videos were provided for reference (http://www.cfsph.iastate.edu/pdf/oral-fluid-collection-in-pigs).

3.3.2 Nucleic Acid Extraction of Oral fluids

DNA and RNA extraction was performed using the BioSprint 96 One-For-All Vet kit (Qiagen Inc., Valencia, California, USA; Catalog# 947057) according to the manufacturer's instructions. For RNA extraction, 100 µl of each OF sample was used and 200 µl of each OF sample used for DNA extraction (PCV2). For the elution step, 70 µl of RNase and 75 µl of DNase-free water was used. Phosphate buffered saline (PBS) was used as the negative extraction control (NEC) and one NEC was prepared with each extraction. Internal control for IAV-S, Armored RNA® Enterovirus, (Ambion Diagnostics Inc., Austin, Texas USA; Asuragen Catalog# 42050) was spiked into lysis buffer mixture prior to RNA extraction. The DNA and RNA were immediately stored at -70°C after extraction. The remaining frozen oral fluid samples were saved for potential future studies.

3.3.3 Nucleic Acid Extraction of Nasal swabs

Each swab was placed in 1.5 ml of Eagle's Minimum Essential Medium with antibiotics, bovine albumin, and TPCK treated trypsin (Gibco, Grand Island, New York USA), in a 3 ml test tube with cap, vortexed for 10 s and transferred to a 2 ml cryovial. Samples were then pooled by 3 or 4 to create a 500 µl-pooled sample for extraction. The RNA was extracted from the pooled sample using the column method, according to manufacturer's instructions (Qiagen; RNeasy Mini Kit Catalog# 74104). For the elution step, 100 µl of RNase and DNase free water was used.

3.3.4 PCR assays

Real-time, Reverse transcription PCR (RRT-PCR) assays were performed to identify PRRSV and IAV-S, and a real-time PCR was performed to identify PCV2 in the OF samples. Primer sets were selected for highly conserved genes for each pathogen. A commercial kit was used for the PRRSV assay (Tetracore Inc., Rockville Maryland USA; Catalog# TC-9060-096). The CFIA designed RRT-PCR protocol for IAV-S was used to test the OF samples. The nasal swabs were tested using a commercially available kit IAV-S kit (Life Technologies, Austin, Texas USA; Catalog# 4415200). An in-house SYBR Green assay was used for the PCV2 assay (McIntosh 2009).

The master mixes were prepared in a clean, 1.5-ml nuclease-free microcentrifuge tubes and reaction tubes were placed in a cooling block while transferring the master mix. Aerosol-resistant pipette tips were used throughout the procedure. Positive controls for the PCR tests were provided by PDS, along with the CFIA IAV-S and PCV2 primer probe sequences (Table 3.1). Internal positive control, positive test control, negative test control and extraction blank were used with each testing batch.

3.3.4.1 Validation of RT-PCR techniques for oral fluids

The real-time PCR amplification efficiency of PRRSV, IAV-S and PCV2 in an OF sample were determined in the previous chapter. These were determined to be: 94.92% (R^2 = 0.99096) and 100.42% (R^2 = 0.99971) for PRRSV OF dilutions for manual extraction and machine extraction respectively. For IAV-S manual extraction, 93.2% (R^2 = 0.99638) and for machine extraction, 76.77% (R^2 = 0.98534) amplification efficiencies were obtained. Moreover, 74.61% (R^2 = 0.9959) and 78.9% (R^2 = 0.9778) RT-PCR efficiencies were determined for manual extraction and machine extraction respectively for PCV-2 OF dilutions.

3.3.4.2 Influenza A subtyping

Samples that were PCR positive for IAV-S were subtyped using a commercial kit (Life Technologies; Catalog# 4445067). The assay was carried out according to manufacturer's instructions with two separate multiplex RRT-PCR assays for H1H3 and N1N2. The primers and probe sets for this assay are proprietary and are not published.

A cycle threshold (C_t) of 35 was considered a cut-off level for each subtype.

3.3.4.3 Influenza A Sequencing

Individual nasal swab samples (3-7) per sampling set, were selected based on a Ct value of 32 or less and subtyped (some sampling sets had pools with different subtypes). These samples were sent to the University of Minnesota Veterinary Diagnostic Laboratory (UMVDL) for virus isolation. At least one virus isolated per sampling set had the hemagglutinin (HA) gene sequenced at UMVDL (Hoffman 2001).

The evolutionary history was inferred using the Neighbor-Joining method (Saitou 1987). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches (Felsenstein 1985). The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura 2004) and are in the units of the number of base substitutions per site. The analysis involved 58 nucleotide sequences. There were a total of 1691 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 (Tamura 2013).

3.3.5 Statistical Analysis

Real-time PCR results were used to obtain binary (positive/negative) outcomes. Data were analyzed at the pen-level and barn-level. Detection frequency for a pathogen was calculated as the number of pens testing positive divided by the total number of pens tested, separately for

each year. Months were grouped into seasons for the analysis: November to December as season 1, January to February as season 2 and March to May as season 3. Barns were considered positive for a pathogen each season if at least one pen tested positive for that pathogen. Barn-level prevalence for a pathogen was calculated as the number of positive barns divided by the total number of barns tested, separately for each year. Additionally, the prevalence for each pathogen over the period was determined.

Detection frequency of PRRSV, IAV-S, and PCV2 was examined for differences by season and by year, accounting for farm clustering. The analyses were done using STATA v13 (STATA Corp., College Station, TX USA). The season and year of oral fluid collection were included in the statistical models as fixed effects. The number of pens testing positive by PCR for PRRSV, IAV-S and PCV2 was the outcome variable and ranged from 0 to 6. Two-level Poisson regression and two-level negative binomial models accounting for clustering by farm were compared. The Likelihood Ratio test was used to compare the goodness of fit of two-level versus single-level regression models. Furthermore, the predicted counts of the two-level Poisson regression and two-level negative binomial models were compared to the raw data (total number of positive pens) across all season and year. Moreover, predicted count rates of PRRSV, IAV-S, and PCV2 by season and year were calculated to determine if temporal changes existed. $P < 0.05$ was considered as statistically significant, and all estimates were presented with 95% confidence interval.

3.4 Results

OF samples were collected from ten farms during the 2013-2014 season. For these ten farms, six farms submitted a full set of 30 samples, and one submitted an extra set in April (n=36). Two farms submitted 12 samples and one farm submitted 17 samples. There was a total of 257

samples collected in year 1. For the 2014-2015 season, seven farms submitted a full set of 30 samples; one farm submitted 24 samples and one farm submitted 18 samples, for a total of 252 samples in year 2. The grand total was 509 out of 600 possible samples collected or 84.8%. Samples were received within 72 hours of collection, were sufficient volume for testing, and were in good condition.

3.4.1 Detection frequency

3.4.1.1 Pen-level results

During the first winter season, 257 OF samples were collected, and 46 (17.90%) were positive for PRRSV, 9 (3.50%) were positive for IAV-S, and 156 (60.70%) were positive for PCV2 (Figure 3.2). During the second winter season, 252 OF samples were collected, and 19 (7.54%) were positive for PRRSV, 16 (6.35%) were positive for IAV-S, and 76 (30.16%) were positive for PCV2 (Figure. 3.2).

3.4.1.2 Barn-level results

The barn-level prevalence of PRRSV, IAV-S and PCV2 were 50%, 30% and 80%, respectively, in the first winter (Figure. 3.3) and 55.5%, 33.3%, and 88.9%, respectively, in the second winter (Figure. 3.3). Furthermore, more than one pathogen was found in six barns, and all three pathogens (PRRSV, IAV-S, and PCV2) were found in one farm in both years.

3.4.2 Temporal analysis

Barn-level prevalence from year 1 to year 2 was similar. However, detection frequency of three pathogens changed over the period of collection and pathogens detected varied barn-to-barn (Figure. 3.4 and 3.5) and month-to-month (Figure. 3.6 and 3.7). In fact, detection frequency for PRRSV and IAV-S showed slight fluctuations over the two winter seasons. Though the detection frequency of PCV2 for 2013-2014 winter showed declining trend, for 2014-2015 winter, it

increased until mid-February and then decreased.

3.4.3 Evaluate the positive frequency of OF and NSSW by sample, barn and month for IAV-S detection

Two by two table analysis was used to examine the positive frequency of OF and NSSW by sample, barn and month. Sample, barn and month had 2.5, 4 and 3.1 higher odds, respectively obtained for NSSW compared to OF samples in year 1, year 2 and both years together, respectively (Table 3.7). However, when two by two table analysis for the individual month of testing, we obtained 4.5 higher odds for NSSW compared to OF samples.

3.4.4 Identification of IAV-S strains circulating in SK

During year 1, NSSW were submitted from four of the five farms that had positive PCR tests for IAV-S. Interestingly, two farms that were negative by oral fluids in the nursery had positive NSSW from suckling piglets. Those IAV-S positive OF were subtyped as H1N1, H1N2 and H3N2 while NSSW were subtyped as pH1N1, H1N1 and H3N2 (Table. 3.2).

During year 2, IAV-S positive OF were subtyped as H3N2, H1N1, and H1N2 while NSSW were subtyped as pH1N1 and H3N2 (Table. 3.3). NSSW were submitted for five of the nine farms that had positive for IAV-S. Two farms that tested PCR negative in OF in the nursery pigs had positive NSSW from suckling piglets. Virus isolation was successful on positive NSSW samples from farm 6, 7, 9 and 11 and sequences were within the following genetic clusters: alpha H1N1, 2009 pandemic H1N1, and Cluster IV H3N2 (Figure. 3.8 and 3.9).

3.4.5 Statistical analysis

Log likelihood (LL) and Akaike information criterion (AIC) values did not differ between the Poisson and Negative Binomial models and the dispersion factor in the Negative Binomial model did not differ from zero ($\alpha=0$; $P>0.05$). Therefore, two-level Poisson regression and two-level

negative binomial models were virtually identical. As there is no dispersion, we selected the two-level Poisson model for further statistical analyses. According to likelihood ratio test, two-level Poisson regression model is preferred over single-level Poisson regression model for PRRSV, IAV-S, and PCV2 ($P < 0.001$ for all LR tests).

According to the two-level Poisson regression model for detection frequency of PRRSV, there were no statistical differences between season of collection ($P = 0.94$); however, the detection frequency differed by year ($P = 0.03$). In fact, there were 0.67 (95% CI -1.20, -0.07) fewer pens on average positive for PRRSV in year 2 compared to year 1. Furthermore, estimated average count rate of PRRSV, which was calculated as the number of positive PRRSV pens in year 2, went down by approximately 50% after accounting for farm clustering.

The two-level Poisson regression model for detection frequency of IAV-S revealed that both season ($P = 0.65$) and year ($P = 0.22$) were not significant. Based on the two-level Poisson regression model for pen prevalence of PCV2, season was not significant ($P = 0.45$) but year was significant (beta -0.91, 95% CI -1.23, -0.60; $P = 0.001$). The estimated average number of PCV2 positive pens was about 60% lower in year 2 versus year 1 after accounting for farm clustering.

3.5 Discussion

OF are increasingly popular as a convenient, reliable and economical diagnostic sample that is easy to collect with minimal training (Prickett 2010b). Since many of the respiratory diseases spread easily among pigs in the same pen, OF are a valuable sample of choice for disease surveillance among large swine herds due to the ease of collecting pen-level samples that represent the overall health of all pigs in the pen. Moreover, the natural behavior of swine is quite helpful for OF collection after weaning. OF sampling is not recommended for nursery

piglets because, at this age, interaction with rope and piglets often does not produce a sufficient sample volume for testing.

We found that detection frequency of three viruses changed over the period of collection and viruses detected varied month to month (Tables 3.4 and 3.6). Interestingly, the count rates differ by year, but not by season for PRRSV and PCV2, based on the statistical analysis. This could be due to the gaps in sampling on some months for some of the farms. Fortunately, we obtained less missing data in year 2 compared to year 1. For the pen-level analysis, some farms had several pens positive for at least one virus every month, whereas a few farms had only sporadic detection of any virus.

In this study, barns were considered positive for a pathogen if at least one pen tested positive for that pathogen in a given month. The overall barn-level prevalence for the whole year (a barn is positive for a pathogen for one or more months), from year 1 to year 2 was similar for all three pathogens (Figure. 3.3). However, the pathogens detected varied month-to-month and the prevalence changed over the period of collection (Table. 3.5 and 3.7). More than one pathogen was detected in 6/10 farms in year 1 and 6/9 farms in year 2, indicating co-infection of these respiratory viruses. According to Opriessnig 2011, an animal infected by a respiratory pathogen is highly susceptible to infection by another respiratory pathogen in the nursery/grower phase of production.

3.5.1 Influenza A subtyping and sequencing

We observed a greater number of positive results in NSSW compared to OF. A negative result indicates that there was no evidence of viral nucleic acid in the OF sample tested, and there are many reasons for false negative RT-PCR results. Substances that are inhibitory to RT-PCR in the sample may cause false negative results (Pricket 2010). Normally OF is a mixture of various

substances and inhibitors are highly likely (Chittick 2011). To confirm false negative results, genome extraction using column method can be conducted which we found to be more sensitive during the validation. Also, RT-PCR tests can be conducted in triplicate samples though it is not practical for a large number of samples. On the other hand, Van Reeth 2012 described that NSSW is the gold standard sample, and it has high sensitivity for respiratory pathogens.

Nasal swabs (NSSW) received with some of the oral fluid samples were tested for IAV-S by RRT-PCR, and positive samples were detected more frequently in NSSW than OF. However, we cannot conclude that one sampling method was better than another in this study because the NSSW were not consistently taken from pigs in the same pens as the OF. Farms that were negative by oral fluids from 8 to 12-weeks-old grower-finisher pigs; however, had positive NSSW results from 14 to 21-days-old suckling piglets that are highly susceptible to diseases. This was due to the fact that some farms knew from experience (outside this study) that they have had more frequent detection of IAV-S in the nursery.

3.5.4 Conclusions

NSSW has a frequency for IAV-S detection compared to OF. Moreover, there was no effect of the season for sampling and currently circulating influenza viruses of these Saskatchewan swine farms are alpha H1N1, 2009 pandemic H1N1, and Cluster IV H3N2.

Table 3.1 Comparison of IAV-S subtyping in OF and NSSW samples in 2013-2014

		Nov	Dec	Jan	Feb	Mar	Apr	May
Farm # 1	OF	NS	0/6	0/6	NS	NS	NS	NS
	NSSW	NS	0/3 3 pools	0/3 3 pools	NS	NS	NS	NS
Farm # 2	OF	0/6	1/6 H3N2	2/6 H3N2	0/6	0/6	0/6	0/6
	NSSW	NS	NS	NS	NS	NS	NS	NS
Farm # 4	OF	0/6	NS	NS	NS	NS	NS	NS
	NSSW	0/2 2 pools	NS	NS	NS	NS	NS	NS
Farm # 5	OF	NS	0/6	NS	NS	NS	NS	NS
	NSSW	NS	0/2 2 pools	NS	NS	NS	NS	NS
Farm # 6	OF	0/6	0/6	0/6	0/6	0/6	0/6	0/6
	NSSW	NS	NS	NS	NS	NS	NS	NS
Farm # 7	OF	0/6	3/6 H3N2 H1N1	0/6	1/6 H3N2 H1N1	1/6 H3N2	0/6	0/6
	NSSW	NS	3/3 H3N2 pH1N1 3 pools	NS	NS	NS	NS	NS
Farm # 9	OF	0/6	0/6	0/6	0/6	0/6	0/6	0/6
	NSSW	3/5 H3N2 H1N1 5 pools	NS	NS	NS	4/4 H1N1 H3N2 4 pools	NS	NS
Farm # 10	OF	1/6 H3N2	0/6	0/6	0/6	0/6	0/6	0/6
	NSSW	NS	NS	0/3 3 pools	NS	NS	NS	NS

NS = No Sample

Table 3.2 Comparison of IAV-S subtyping in OF and NSSW samples in 2014-2015

		Nov	Dec	Jan	Feb	Mar	Apr
Farm # 2	OF	0/6	0/6	0/6	0/6	0/6	NS
	NSSW	NS	NS	0/3 3 pools	NS	1/3 3 pools	NS
Farm # 3	OF	NS	0/6	0/6	NS	NS	NS
	NSSW	NS	0/5 5 pools	0/3 3 pools	NS	NS	NS
Farm # 4	OF	0/6	NS	0/6	0/6	NS	NS
	NSSW	3/3 3 pools	NS	3/3 3 pools	3/3 3 pools	NS	NS
Farm # 6	OF	0/6	0/6	0/6	0/6	1/6 H3N2	0/6
	NSSW	3/3 H1N1 3 pools	2/3 pH1N1 3 pools	0/3 3 pools	NS	1/3 H1N1 3 pools	NS
Farm # 7	OF	0/6	1/7 H3N2	0/6	0/6	0/6	0/6
	NSSW	NS	1/3 H3N2 3 pools	NS	0/3 3 pools	NS	NS
Farm # 8	OF	0/6	0/6	0/6	0/6	0/6	0/6
	NSSW	0/3 3 pools	0/3 3 pools	0/3 3 pools	NS	NS	NS
Farm # 9	OF	0/6	0/6	0/6	0/6	0/6	0/6
	NSSW	3/3 H1N1 H1N2 3 pools	3/3 H3N2 3 pools	2/3 3 pools	3/3 H3N2 3 pools	3/3 3 pools	NS
Farm # 11	OF	4/6 H3N2 H1N2	2/6 H1N1 H1N2	4/6 H3N2 H1N2	2/6 H3N2 H1N2	2/6 H3N2 H1N2	0/6
	NSSW	4/4 H3N2 4 pools	2/3 H1N1 3 pools	3/3 H3N2 3 pools	3/3 H3N2 H1N2 3 pools	3/3 H3N2 H1N2 3 pools	NS
Farm # 12	OF	0/6	0/6	0/6	0/6	0/6	0/6
	NSSW	0/3 3 pools	0/3 3 pools	0/3 3 pools	0/3 3 pools	0/3 3 pools	NS

NS = No Sample

Table 3.3 Detection frequency of PRRS, IAV-S and PCV2 by month in 2013-2014 as a proportion of total number of samples.

	PRRS	IAV-S	PCV2
November	15/48	0/48	30/48
December	7/36	1/36	30/36
January	4/47	6/47	26/47
February	8/36	0/36	25/36
March	5/48	1/48	24/48
April	6/36	1/36	15/36
May	1/6	0/6	6/6

Table 3.4 Barn-level prevalence of PRRS, IAV-S and PCV2 by month in 2013-2014 as a proportion of total number of samples.

	PRRS	IAV-S	PCV2
November	4/8	0/8	7/8
December	2/6	1/6	6/6
January	2/8	3/8	6/8
February	3/6	0/6	6/6
March	1/8	1/8	5/8
April	3/6	1/6	4/6
May	1/1	0/1	1/1

Table 3.5 Detection frequency of PRRS, IAV-S and PCV2 by month in 2014-2015 as a proportion of total number of samples.

	PRRS	IAV-S	PCV2
November	5/54	4/54	9/54
December	1/48	3/48	14/48
January	3/48	4/48	18/48
February	4/54	2/54	20/54
March	5/42	3/42	15/42
April	1/6	0/6	0/6
May	0/0	0/0	0/0

Table 3.6 Barn-level prevalence of PRRS, IAV-S and PCV2 by month in 2014-2015 as a proportion of total number of samples.

	PRRS	IAV-S	PCV2
November	3/9	1/9	5/9
December	1/8	2/8	5/8
January	1/8	1/8	5/8
February	2/9	1/9	7/9
March	1/7	2/7	5/7
April	1/1	0/1	0/1
May	0/0	0/0	0/0

Table 3.7 Evaluates the positive frequency of OF and NSSW by sample, barn and month for IAV-S detection.

YEAR 1	OF	NSSW			
Barn positive	1	2	3	Exact test	Odds(NS) = 0.5
Barn negative	5	4	9	P=1.0	Odds(OF) = 0.2
	6	6			OR (NS>OF0) = 2.5

YEAR 2	OF	NSSW			
Barn positive	3	6	9		Odds(NS) = 2
Barn negative	6	3	9	P=.35	Odds(OF) = 0.5
	9	9			OR (NS>OF0) = 4

YEAR 1+2	OF	NSSW			
Barn positive	4	8	12		Odds(NS) = 1.1
Barn negative	11	7	18	P=.26	Odds(OF) = 0.4
	15	15			OR (NS>OF0) = 3.1

BARN MONTHS	OF	NSSW			
Barn positive	8	21	29		Odds(NS) = 1.2
Barn negative	31	18	49	P=.005	Odds(OF) = 0.3
	39	39			OR (NS>OF0) = 4.5

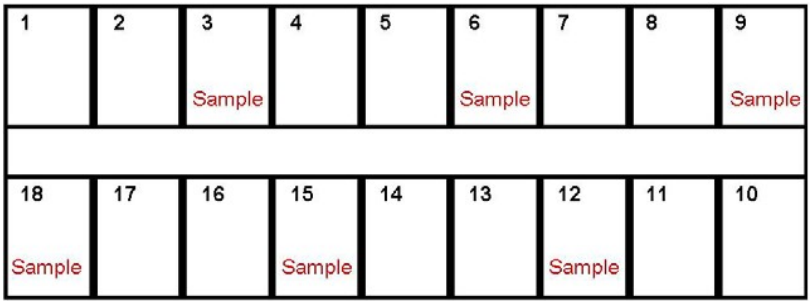

Figure 3.1 A diagram of sampling stratagem in a barn.

The diagram shows a barn with 18 pens within a single room to demonstrate how a person may spread out the oral fluid sampling within the room.

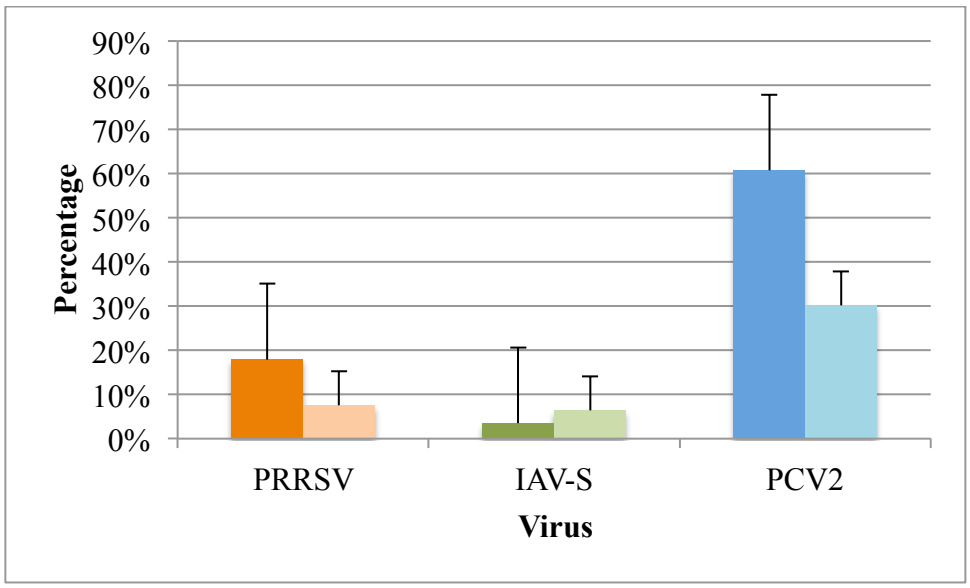

Figure 3.2 Overall detection frequency for PRRSV, IAV-S and PCV2 (darker bars shows November 2013 to May 2014 and lighter bars shows November 2014 to May 2015). Error bars indicate standard error.

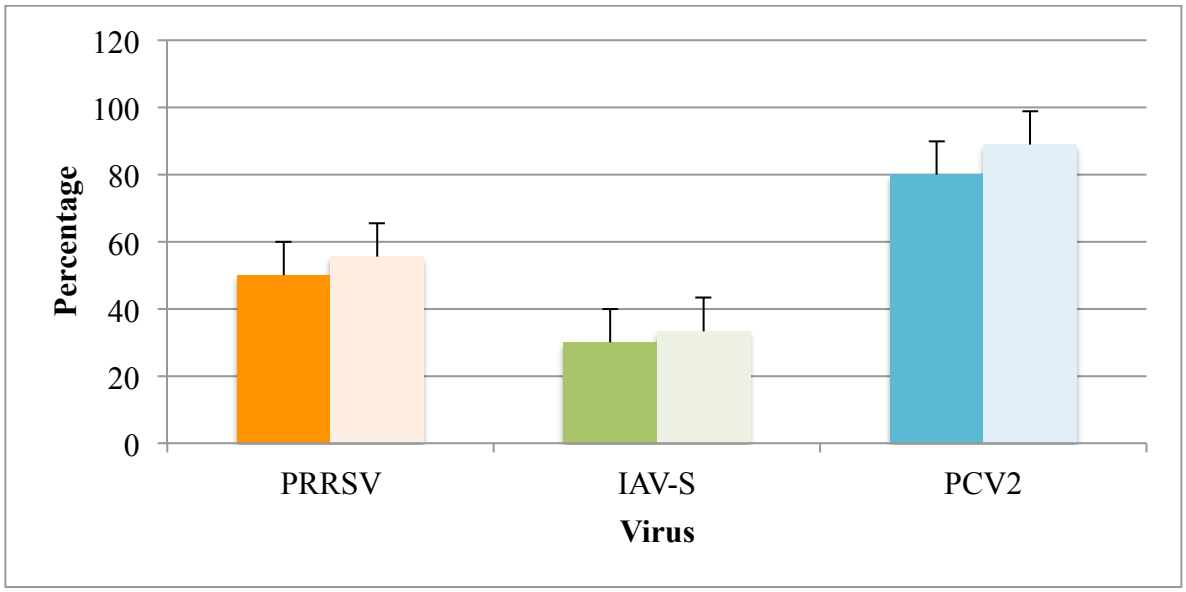

Figure 3.3 Overall barn-level prevalence for influenza A, PRRSBV and PCV2 (darker bars show November 2013 to May 2014 and lighter bars show November 2014 to May 2015). Error bars indicate standard error.

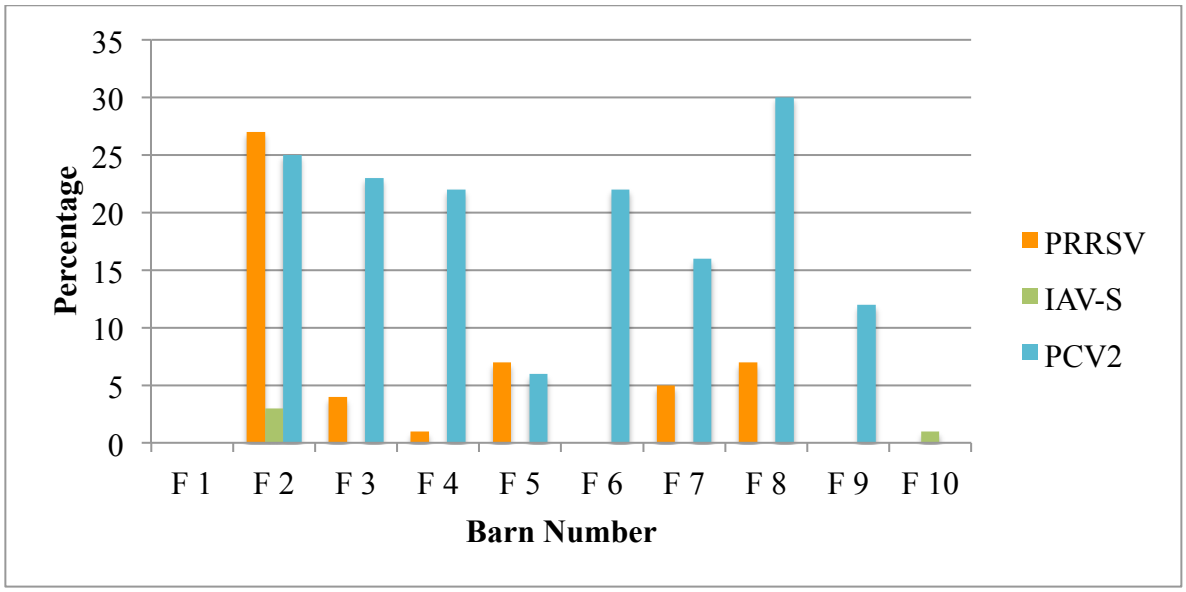

Figure 3.4 Detection frequency for PRRSV, IAV-S, and PCV2 across ten farms (November 2013 to May 2014).

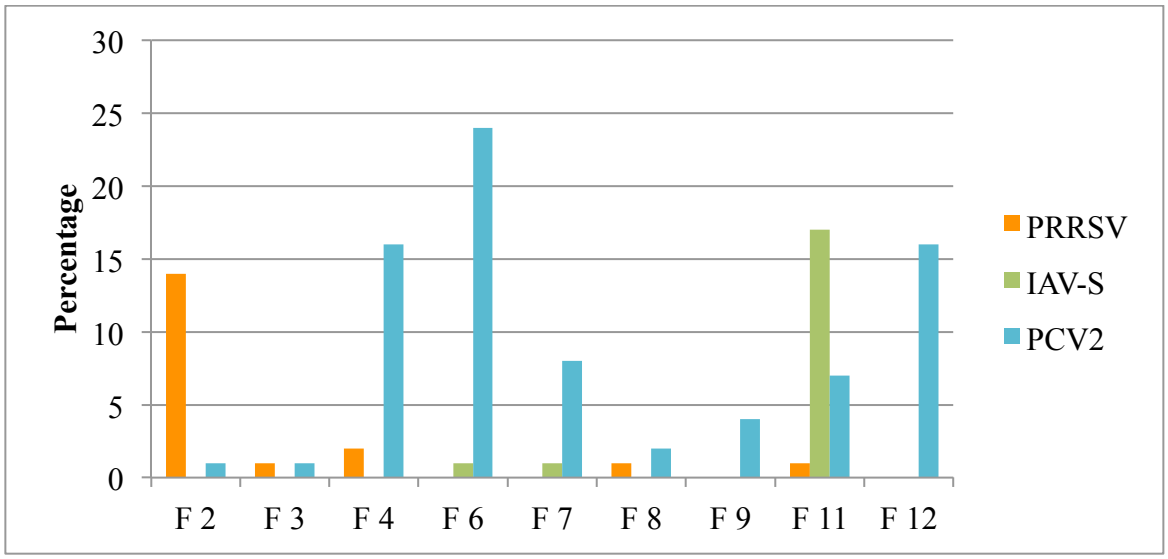

Figure 3.5 Detection frequency for PRRSV, IAV-S, and PCV2 across ten farms (November 2014 to May 2015).

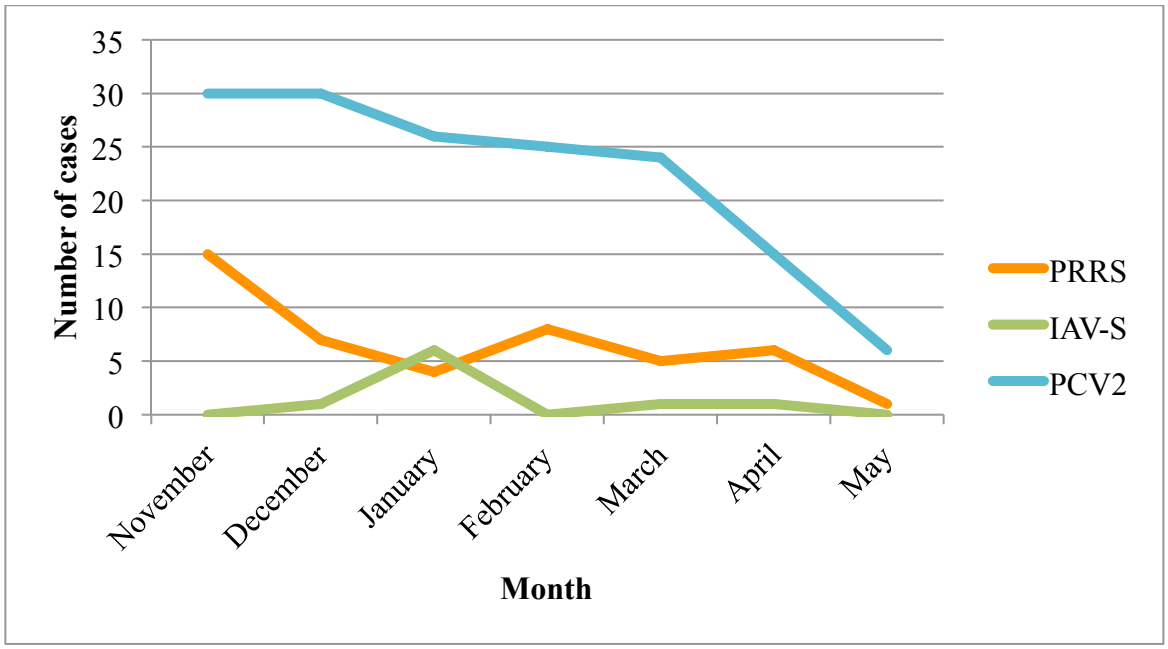

Figure 3.6 Detection frequency for PRRSV, IAV-S, and PCV2 across seven months (November 2013 to May 2014).

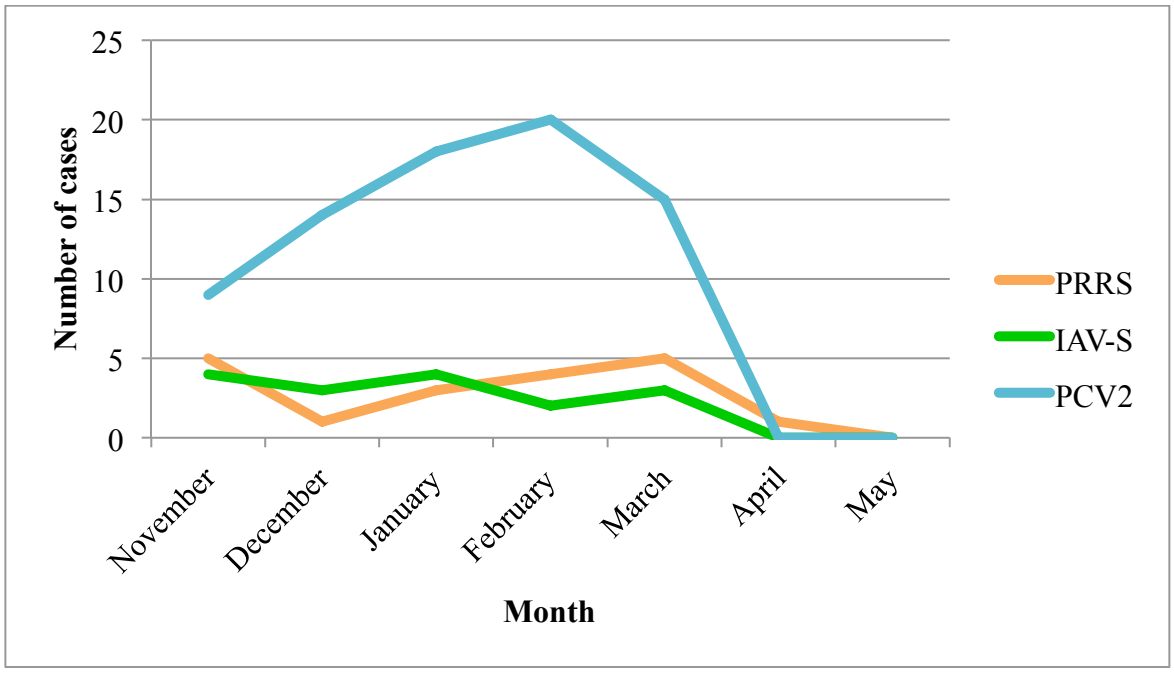

Figure 3.7 Detection frequency for PRRSV, IAV-S, and PCV2 across seven months (November 2014 to May 2015).

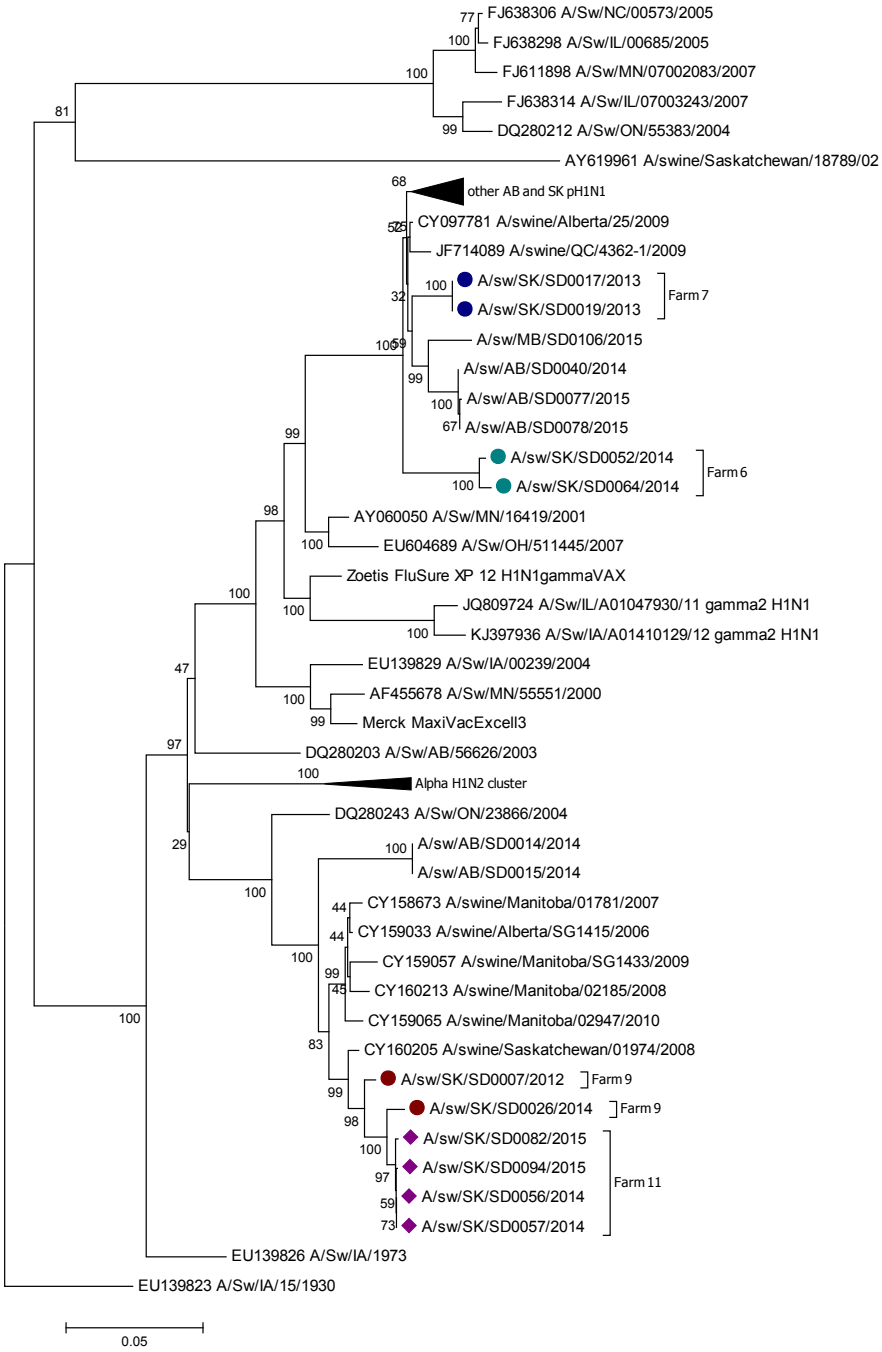

Figure 3.8 Evolutionary relationships of influenza A virus, H1 subtypes.
The evolutionary history was inferred using the Neighbor-Joining method, computed using the Maximum Composite Likelihood method and conducted in MEGA6. Bootstrapping was done using 500 replicates and 1691 nucleotide positions.

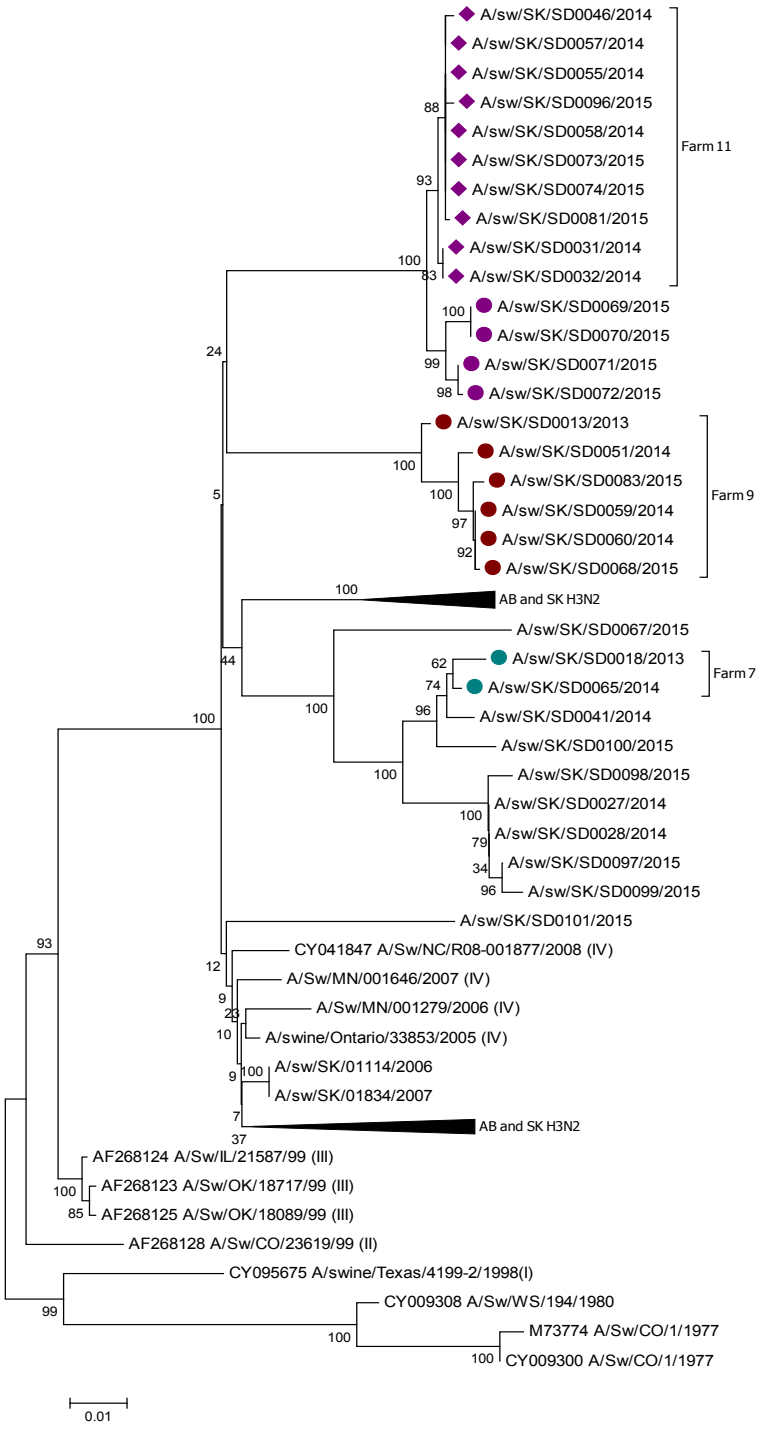

Figure 3.9 Evolutionary relationships of influenza A virus, H3 subtypes.
The evolutionary history was inferred using the Neighbor-Joining method, computed using the Maximum Composite Likelihood method and conducted in MEGA6. Bootstrapping was done using 500 replicates and 1392 nucleotide positions.

4 GENERAL DISCUSSION

4.1 Discussion

Detection of PRDC pathogens in oral fluids (OF) by real-time PCR is fast, popular and convenient method and it has both benefits and some limitations. In fact, OF is an easy, reliable, safe, and non-invasive diagnostic sample (Detmer 2011, Escribano 2012, Gutierrez 2012, Prickett 2010b, Romagosa 2012, Seddon 2012). Additionally, it is a pooled sample that represents the health status of many pigs.

However, OF has limitations as a diagnostic sample. According to Detmer (2011), human oral fluid has inhibitors and neutralizers against IAV strains and swine OF might contain them though there have been limited investigations into these compounds. Furthermore, the environmental temperature may affect the viability of infectious agent in the sample, and the composition of OF is not identical to serum. However, according to Kittawornrat (2012), diagnostic sensitivity and specificity are high in detecting PRRSV antibodies in oral fluid samples using commercial serum antibody enzyme-linked immunosorbent assay (ELISA) kit.

Despite these limitations, the ease of use and accessibility of OF make it a good sample for surveillance when used with highly sensitive PCR assays for PRRSV, IAV-S, and PCV2. Therefore, in this study, we use swine OF to detect PRDC pathogens by real-time PCR.

4.2 Limitations

In my surveillance study, there was an experimental design limitation since the age of sampling did not match the age of shedding for IAV-S on all farms. On farms using sow vaccinations for IAV-S, the virus is often more readily detected in the 6 to 10-week-old pigs, compared to farms that do not use IAV-S vaccinations where suckling piglets are more likely to be shedding IAV-S (Detmer, personal communication).

The OF were collected on 8 to 12-week-old grower-finisher pigs; however, NSSW for IAV-S were collected from 14 to 21-day-old suckling piglets. While grower-finisher pigs may be infected with IAV-S, there appears to be a higher quantity of virus in the sample, and longer shedding in pigs around weaning age (Detmer 2013a). For example, during the 2014-2015 winter season on farm #9, which we know to be endemically infected with both alpha H1N1 and group IV H3N2 viruses, there were no positive OF samples out of the 30 pens tested over the five month period. During the same period (sampled on the same day, but on 14-21 day old animals instead of 10-week-old pigs for OF), there were 41 positive (NSSW) samples out of 50 and the farm was considered positive five months out of five. Further, RNA extraction was redone using the manual method (which had higher efficiency during the validation process in Chapter 2) on the above mentioned 30 OF samples collected in 2014-2015 and retested the new extracts for IAV-S. The results for the five months (November to March) were: 1/6, 0/6, 0/6, 2/6, and 2/6. These results still detected the virus less often than the NSSW but were higher than with the machine extraction (5 positive pens compared to 0).

Another issue that we had on farm #9 was two months with false positive results for PRRSV. This farm is PRRSV-negative, and serum ELISA tests were conducted in January 2015 to confirm the negative status of this farm. When these samples were retested using manual extraction, the results were negative.

These results bring up the importance of optimization of extraction methods and PCR assays for diagnostic tests. In this study, we performed machine genome extraction by BioSprint 96 workstation because we were testing about 600 samples (up to 90 samples at one time). One of the main differences between manual and machine extraction is the volume of the OF sample that used for extraction; 500 µl OF was used for manual extraction while 100 µl sample volume

used for machine extraction of RNA. However, 200 µl sample volume was used for both manual and machine DNA extraction in the PCV2 protocol. Therefore, it is less likely to extract more genome by machine extraction for IAV-S and PRRSV protocol and more likely to have higher false-negative results.

The protocol that we received from the University of Minnesota Veterinary Diagnostic Laboratory (UMVDL) for the BioSprint 96 workstation uses 200 µl of the sample for RNA extraction from OF. The UMVDL IAV-S assay had higher sensitivity for the same viruses we spiked our OF with during the validation process (Detmer 2013b). Therefore, there is an opportunity to optimize the extraction method that we used to improve our analytical sensitivity.

The laboratory-to-laboratory variation of oral fluid antibody testing by enzyme-linked immunosorbent assay (ELISA) has been examined for PRRSV in a ring test **(**Kittawornrat 2012). Moreover, Chittick 2011 has compared different RNA extraction and RT-PCR methods to detect PRRSV RNA in OF samples, and the results show the importance of further optimization.

Mycoplasma hyopneumoniae (Mhyo) is an important bacterial agent associated with PRDC and it contributes to significant economic losses worldwide. Due to the importance of knowing the prevalence of Mhyo, we tried to validate a real time PCR protocol for Mhyo to use in this assay. Using a pure bacterial isolate of Mhyo provided by the University of Guelph, tenfold dilution series were prepared. DNA was manually extracted (Qiagen; DNeasy blood and tissue kit Catalog# 69506) and real time PCR was conducted using a commercial kit (Life Technologies; VetMAX™ *M. Hyopneumoniae* Catalog# 4415217 and 4415198). Only the undiluted sample had a positive result; all dilutions were negative. Furthermore, the same DNA extracts were also tested using conventional PCR technique with the same results (Woolley 2012, Strait 2008).

Moreover, in this study, farm selection was done by a convenience selection method. Meaning that the farms were not randomly selected, rather by the presence of at least one of the pathogens, willingness to participate and ability to collect and submit the samples. Therefore, the results from these selected farms may not be a true indicator of PRDC prevalence status of Saskatchewan. To accurately estimate the prevalence in Saskatchewan province, an epidemiological approach of farm selection should be used. However, the data collected in this study can be used to calculate sample size for such a study. Additional work would also be required to determine the best sampling strategy for calculating the pen-level prevalence in a future study.

In the overall surveillance study, only 25 OF samples among 509 OF samples were positive for IAV-S for both sampling years, and this IAV-S prevalence was lower than expected. This indicates the number of samples that we collected were not enough to calculate the IAV-S prevalence accurately. On the other hand, the prevalence for PCV2 was comparatively higher, and this indicates the number of samples that we collected were higher than the optimum number. Thus, this pilot study will help to calculate the optimal number of OF samples to calculate the accurate prevalence of each pathogen.

4.3 Future directions

The results of this pilot project on surveillance of pigs using OF provides a starting point for a larger, more extensive project. To take this work further and develop a full study on the prevalence of PRDC, several things must be done. Optimization of IAV-S, PRRSV, and PCV2 validation protocols and optimization of the nucleic acid extraction would be vital to using these PCR protocols for a new study. Selection of the optimal age range for sampling, selection of a

suitable sample of choice, test sample duplicates or triplicates, and collection of an optimal number of samples, should also be done.

According to Kittawornrat 2012, precision of PRRSV oral fluid antibody ELISA was assessed for assay repeatability and reproducibility. This ring test evaluation is important for obtaining the precision of routine diagnostic testing. Therefore, IAV-S, PRRSV, and PCV2 PCR protocols must be optimized further by different technicians and in different laboratories to detect the assay repeatability and reproducibility respectively.

Additionally, further development and optimization work should be undertaken to improve the machine based extraction protocol for diagnostic laboratories since manual extraction is not a practical application for busy laboratories. According to Goodell 2014, the gold standard antemortem sample for swine respiratory diseases is nasal swabs. The sensitivity of nasal swabs is considerably higher than OF, and it would be a great sample of choice for diagnostic studies Moreover, it is essential to prepare and test duplicate or triplicate technical replications of each dilution to ensure high accuracy.

Additionally, selection of the optimal age range for sampling is crucial. For instance, piglets are highly susceptible to respiratory diseases in their first 2-3 weeks of lives (Panyasing 2014). If samples were collected during this period more IAV-S, positive samples might be detected compared to if we collect samples in older pigs. However, for oral fluids, it has already be demonstrated that the ten week age range is better for PCV2 and PRRS detection (Kittawornrat 2012). Furthermore, it is essential to calculate Mhyo prevalence using an optimized real-time protocol as Mhyo is a significant PRDC pathogen.

As an economically important disease complex, there is a necessity to launch Canada-wide PRDC pathogen surveillance. To launch a cross-sectional study, the disease should be prevalent

in the population (Pearce 2004). In this case, it is well known that PRDC pathogens are highly prevalent in Canadian swine herds. To initiate such prevalence study, estimation of the sample size, determinations of farms and practical issues to overcome are important considerations.

Estimation of sample size is an essential factor, and it is necessary to have a good idea about the population size. The actual population size can be determined by conducting a census and if it is not achievable one can make an educated guess after reviewing literature properly. If the population is considerably large, a subpopulation can be used as the sample, and it should be representative. The optimal sample size is determined by the precision that is required, sampling method and the actual variability of the population (Chadha 2006). The sample size can be calculated by using formulae (Arya 2012, Naing 2006), ready-made table (Lwanga 1991), nomograms or computer software (Chadha 2006). In a descriptive study, it is essential to decide the level of confidence (CI) that is commonly set as 95% and the power of the study, which is related to the size of the sample (Dohoo 2009). Additionally, convenient or random sampling methods are more useful in prevalence studies.

There are some challenging issues related to a surveillance study such as financial problems (Chadha 2006), missing data and extreme prevalence (Arya 2012). In fact, if the funds are fixed, the higher the cost per sample, the smaller the size of the sample. Moreover, it is essential to minimize the missing data as much as possible.

Taking all the results into consideration, the baseline results of this pilot project will be a useful tool to predict the total number of samples to calculate the overall prevalence of PRRSV, IAV-S, and PCV2 in SK. Additionally, OF will be a reasonable sample of choice on farms with low PRDC prevalence, and real-time PCR will be an effective technique to detect PRDC pathogens after additional assay optimization.

5 REFERENCES

Allan, G.M., McNeilly, F., Kennedy, S., Daft, B., Clarke, E.G., Ellis, J.A., Haines, D.M., Meehan, B.M. & Adair, B.M. (1998) Isolation of porcine circovirus-like viruses from pigs with a wasting disease in the USA and Europe. *J Vet Diagn Invest*, **10**(1), 3-10.

Allerson, M., Deen, J., Detmer, S.E., Gramer, M.R., Joo, H.S., Romagosa, A. & Torremorell, M. (2013) The impact of maternally derived immunity on influenza A virus transmission in neonatal pig populations. *Vaccine*, **31**(3), 500-505.

Arya, R., Antonisamy, B. & Kumar, S. (2012) Sample size estimation in prevalence studies. *Indian J Pediatr*, **79**(11), 1482-1488.

Bateman, A.C., Kieke, B.A., Irving, S.A., Meece, J.K., Shay, D.K. & Belongia, E.A. (2013) Effectiveness of monovalent 2009 pandemic influenza A virus subtype H1N1 and 2010-2011 trivalent inactivated influenza vaccines in Wisconsin during the 2010-2011 influenza season. *J Infect Dis*, **207**(8).

Benfield, D.A., Nelson, E., Collins, J.E., Harris, L., Goyal, S.M., Robison, D., Christianson, W.T., Morrison, R.B., Gorcyca, D. & Chladek, D. (1992) Characterization of swine infertility and respiratory syndrome (SIRS) virus (isolate ATCC VR-2332). *J Vet Diagn Invest*, **4**(2), 127-133.

Botner, A., Strandbygaard, B., Sorensen, K.J., Have, P., Madsen, K.G., Madsen, E.S. & Alexandersen, S. (1997) Appearance of acute PRRS-like symptoms in sow herds after vaccination with a modified live PRRS vaccine. *Vet Rec*, **141**(19), 497-499.

Brockmeier, S.L., Palmer, M.V., Bolin, S.R. & Rimler, R.B. (2001) Effects of intranasal inoculation with Bordetella bronchiseptica, porcine reproductive and respiratory syndrome virus, or a combination of both organisms on subsequent infection with Pasteurella multocida in pigs. *Am J Vet Res.* **62**(4), 521–525.

Broeders, S., Huber, I., Grohmann, L., Berben, G., Taverniers, I., Mazzara, M., Roosens, N. & Morisset, D. (2014) Guidelines for validation of qualitative real-time PCR methods. *Trends Food Sci Technol.* 37, 115-126.

Burd, E.M. (2010) Validation of laboratory-developed molecular assays for infectious diseases. *Clin Microbiol Rev*, **23**(3), 550-576.

Burkardt, H.J. (2000) Standardization and quality control of PCR analyses. *Clin Chem Lab Med*, **38**(2), 87-91.

Bustin, S.A. & Nolan, T. (2004) Pitfalls of quantitative real-time reverse-transcription polymerase chain reaction. *J Biomol Tech*, **15**(3), 155-166.

Chadha, V.K. (2006) Sample size determination in health studies. *NTI Bulletin*, **42**(3&4), 55 –

62.

Chae, C. (2004) Postweaning multisystemic wasting syndrome: a review of etiology, diagnosis and pathology. *Vet J*, **168**(1), 41-49.

Chand, R.J., Trible, B.R. & Rowland, R.R. (2012) Pathogenesis of porcine reproductive and respiratory syndrome virus. *Curr Opin Virol*, **2**(3), 256-263.

Chang, C.C., Chung, W.B., Lin, M.W., Weng, C.N., Yang, P.C., Chiu, Y.T., Chang, W.F. & Chu, R.M. (1993) Porcine reproductive and respiratory syndrome (PRRS) in Taiwan. I. Viral isolation. *J. Chin. Soc. Vet. Sci*, **19**, 268–276.

Chittick, W.A., Stensland, W.R., Prickett, J.R., Strait, E.L., Harmon, K., Yoon, K.J., Wang, C. & Zimmerman, J.J. (2011) Comparison of RNA extraction and real-time reverse transcription polymerase chain reaction methods for the detection of porcine reproductive and respiratory syndrome virus in porcine oral fluid specimens. *J Vet Diagn Invest*, **23**(2), 248-253.

Cortey, M., Napp, S., Alba, A., Pileri, E., Grau-Roma, L., Sibila, M. & Segales, J. (2011) Theoretical and experimental approaches to estimate the usefulness of pooled serum samples for the diagnosis of postweaning multisystemic wasting syndrome. *J Vet Diagn Invest*, **23**(2), 233-240.

Corthier, G. (1976) Swine fever: influence of passive immunity on pig immune response following vaccination with a live virus vaccine (Thiverval strain). *Ann Rech Vet*, **7**(4), 361-372.

Christianson, W.T., Choi, C.S., Collins, J.E., Molitor, T.W., Morrison, R.B. & Joo, H.S. (1993) Pathogenesis of porcine reproductive and respiratory syndrome virus infection in mid-gestation sows and fetuses. *Can J Vet Res*, **57**(4), 262-268.

Decorte, I., Van der Stede, Y., Nauwynck, H., De Regge, N. & Cay, A.B. (2013) Effect of saliva stabilisers on detection of porcine reproductive and respiratory syndrome virus in oral fluid by quantitative reverse transcriptase real-time PCR. *Vet J*, **197**(2), 224-228.

Detmer, S., Gramer, M., Goyal, S., Torremorell, M. & Torrison, J. (2013a) Diagnostics and surveillance for Swine influenza. *Curr Top Microbiol Immunol*, **370**, 85-112.

Detmer, S.E., Gunvaldsen, R.E. & Harding, J.C. (2013b) Comparison of influenza A virus infection in high- and low-birth-weight pigs using morphometric analysis. *Influenza and Other Respiratory Viruses*, **7** (4), 2–9.

Detmer, S.E., Patnayak, D.P., Jiang, Y., Gramer, M.R. & Goyal, S.M. (2011) Detection of Influenza A virus in porcine oral fluid samples. *J Vet Diagn Invest*, **23**(2), 241-247.

Dietze, K., Pinto J., Wainwright, S. & Hamilton, C. (2011). Porcine reproductive and respiratory syndrome (PRRS): virulence jumps and persistent circulation in Southeast Asia. In Focus on..., 5, 8. Rome. Food and Agriculture Organization of the United Nations.

Dohoo, I., Martin, W. & Stryhn, H. (2009) Sampling. In *Veterinary Epidemiologic Research*, 2nd Edn (33-53). Charlottetown, PEI: VER Inc.

Dupont, K., Nielsen, E.O., Baekbo, P. & Larsen, L.E. (2008) Genomic analysis of PCV2 isolates from Danish archives and a current PMWS case-control study supports a shift in genotypes with time. *Vet Microbiol*, **128**(1-2), 56-64.

Ellis, J., Hassard, L., Clark, E., Harding, J., Allan, G., Willson, P., Strokappe, J., Martin, K., McNeilly, F., Meehan, B., Todd, D. & Haines, D. (1998) Isolation of circovirus from lesions of pigs with postweaning multisystemic wasting syndrome. *Can Vet J*, **39**(1), 44-51.

Escribano, D., Gutierrez, A.M., Martinez Subiela, S., Tecles, F. & Ceron, J.J. (2012) Validation of three commercially available immunoassays for quantification of IgA, IgG, and IgM in porcine saliva samples. *Res Vet Sci*, **93**(2), 682-687.

Feitsma, H., Grooten, H.J., Schie, F.W.V. & Colenbrander, B. (1992) The effect of porcine epidemic abortion and respiratory syndrome (PEARS) on sperm production. *Proc 12th Intl Congr Anim Reprod*, **12**,1710–1712.

Felsenstein, J. (1985) Confidence Limits on Phylogenies: An Approach Using the Bootstrap. Evolution, 39(4), 783-791. http://www.jstor.org/stable/2408678.pdf accessed on 10 March2009.

Giménez-Lirola, L.G., Xiao, C.T., Zavala, M., Halbur, P.G. & Opriessnig, T. (2013) Improving ante mortem diagnosis of Erysipelothrix rhusiopathiae infection by use of oral fluids for bacterial, nucleic acid, and antibody detection. *J Microbiol Methods*, **92**(2), 113-121.

Goodell, C.K., Prickett, J., Kittawornrat, A., Johnson, J., Zhang, J., Wang, C. & Zimmerman, J.J. (2014) Evaluation of Screening Assays for the Detection of Influenza A Virus Serum Antibodies in Swine. *Transbound Emerg Dis*, **166**, 450-460.

Grau-Roma, L., Fraile, L., Segalés, J. (2011) Recent advances in the epidemiology, diagnosis and control of diseases caused by porcine circovirus type 2. *Vet. J*, **187**(1), 23–32.

Gutierrez, A.M., Ceron, J.J., Fuentes, P., Montes, A. & Martinez-Subiela, S. (2012) Longitudinal analysis of acute-phase proteins in saliva in pig farms with different health status. *Animal*, **6**(2), 321-326.

Hamel, A.L., Lin, L.L. & Nayar, G.P. (1998) Nucleotide sequence of porcine circovirus associated with postweaning multisystemic wasting syndrome in pigs. *J Virol*, **72**(6), 5262-5267.

Hansen, M.S., Pors, S.E., Jensen, H.E., Bille-Hansen, V., Bisgaard, M., Flachs, E.M. & Nielsen, O.L. (2010) An investigation of the pathology and pathogens associated with porcine respiratory disease complex in Denmark. *J Comp Pathol*, **143**(2-3), 120-131.

Harding, J.C. (2004) The clinical expression and emergence of porcine circovirus 2. *Vet Microbiol*, **98**(2), 131-135.

Harding, J.C., Clark, E.G. & Strokappe, J.H. (1998) Postweaning multisystemic wasting syndrome: epidemiology and clinical presentation. *J. Swine Health Prod*, **6(**6**)**, 249–254.

Harding, J.C., Ellis, J.A., McIntosh, K.A. & Krakowka, S. (2010) Dual heterologous porcine circovirus genogroup 2a/2b infection induces severe disease in germ-free pigs. *Vet Microbiol*, **145**(3-4), 209-19.

Harding, J.C.S. & Edward, G.C. (1997) Recognizing and diagnosing postweaning multisystemic wasting syndrome (PMWS). *J. Swine Health and Prod***, 5(**5**),** 201-203.

Harding, J.C.S. (1996) Postweaning multisystemic wasting syndrome: Preliminary epidemiology and clinical findings. *Proc Western Can Assoc Swine Pract*, 21.

Holtkamp, D.J., Kliebenstein, J.B., Neumann, E.J., Zimmerman, J., Rotto, H.F., Yoder, T.K, Wang, C., Yeske, P.E., Mowrer, C.L. & Haley, C.A. (2013) Assessment of the economic impact of porcine reproductive and respiratory syndrome virus on United States pork producers. *J Swine Health Prod*, **21**, 72-84.

Hoffmann, E., Stech, J., Guan, Y., Webster, R.G & Perez, D.R. (2001) Universal primer set for the full-length amplification of all influenza A viruses. *Arch Virol*, **146**(12), 2275-89.

Janke, B.H. (2014) Influenza A virus infections in swine: pathogenesis and diagnosis. *Vet Pathol*, **51**(2), 410-426.

Johnson, W., Roof, M., Vaughn, E., Christopher-Hennings, J., Johnson, C.R. & Murtaugh, M.P. (2004) Pathogenic and humoral immune responses to porcine reproductive and respiratory syndrome virus (PRRSV) are related to viral load in acute infection. *Vet Immunol Immunopathol*, **102**(3), 233-247.

Karasin, A.I., Schutten, M.M., Cooper, L.A., Smith, C.B., Subbarao, K., Anderson, G.A., Carman, S. & Olsen, C.W. (2000) Genetic characterization of H3N2 influenza viruses isolated from pigs in North America, 1977-1999: evidence for wholly human and reassortant virus genotypes. *Virus Res*, **68**(1), 71-85.

Karniychuk, U.U., Geldhof, M., Vanhee, M., Van Doorsselaere, J., Saveleva, T.A. & Nauwynck, H.J. (2010) Pathogenesis and antigenic characterization of a new East European subtype 3 porcine reproductive and respiratory syndrome virus isolate. *BMC Vet Res*, **6**, 30.

Kekarainen, T., McCullough, K., Fort, M., Fossum, C., Segales, J. & Allan, G.M. (2010) Immune responses and vaccine-induced immunity against porcine circovirus type 2. *Vet Immunol Immunopathol*, **136**(3-4), 185-193.

Kim, J., Chung, H.K. & Chae, C. (2003) Association of porcine circovirus 2 with porcine respiratory disease complex. *Vet J*, **166**(3), 251-256.

Kittawornrat, A., Prickett, J., Wang, C., Olsen, C., Irwin, C., Panyasing, Y., Ballagi, A., Rice, A., Main, R., Johnson, J., Rademacher, C., Hoogland, M., Rowland, R. & Zimmerman, J. (2012) Detection of Porcine reproductive and respiratory syndrome virus (PRRSV)

antibodies in oral fluid specimens using a commercial PRRSV serum antibody enzyme-linked immunosorbent assay. *J Vet Diagn Invest*, **24**(2), 262-269.

Krakowka, S., Ellis, J.A., Meehan, B., Kennedy, S., McNeilly, F. & Allan, G. (2000) Viral wasting syndrome of swine: experimental reproduction of postweaning multisystemic wasting syndrome in gnotobiotic swine by coinfection with porcine circovirus 2 and porcine parvovirus. *Vet Pathol*, **37**(3), 254-263.

Ladinig, A., Detmer, S.E., Clarke, K., Ashley, C., Rowland, R.R., Lunney, J.K. & Harding, J.C. (2015) Pathogenicity of three type 2 porcine reproductive and respiratory syndrome virus strains in experimentally inoculated pregnant gilts. *Virus Res*, **203**, 24-35.

Lamb, R.A. & Krug, R.M. (2013) Orthomyxoviridae: The Viruses and Their Replication. In Knipe, D.M. (Ed) *Fields Virology, Volume 2,* 4th Edn (2270-2339)**.** Philadelphia, PA: Lippincott Williams & Wilkins Publishers.

Larochelle, R., Bielanski, A., Muller, P. & Magar, R. (2000) PCR detection and evidence of shedding of porcine circovirus type 2 in boar semen. *J Clin Microbiol*, **38**(12), 4629-4632.

Leuwerke, B. & Loula, T. (2008) Swine influenza: New challenges with old disease. Feedstuffs-The weekly newspaper for agribusiness, **80**(21).

Liew, F.Y., Russell, S.M., Appleyard, G., Brand, C.M. & Beale, J. (1984) Cross-protection in mice infected with influenza A virus by the respiratory route is correlated with local IgA antibody rather than serum antibody or cytotoxic T cell reactivity. *Eur J Immunol*, **14**(4), 350-356.

Linhares, D.C., Cano, J.P., Wetzell, T., Nerem, J., Torremorell, M. & Dee, S.A. (2012) Effect of modified-live porcine reproductive and respiratory syndrome virus (PRRSv) vaccine on the shedding of wild-type virus from an infected population of growing pigs. *Vaccine*, **30**(2), 407-413.

Lorusso, A., Vincent, A.L., Harland, M.L., Alt, D., Bayles, D.O., Swenson, S.L., Gramer, M.R., Russell, C.A., Smith, D.J., Lager, K.M. & Lewis, N.S. (2011) Genetic and antigenic characterization of H1 influenza viruses from United States swine from 2008. *J Gen Virol*, **92**(Pt 4), 919-930.

Lwanga, S.K, & Lemeshow, S. (1991) Sample size determination in health studies: A practical manual.http://apps.who.int/iris/bitstream/10665/40062/1/9241544058_(p1-p22). pdf, access on 13 December 2015.

McIntosh , K. A., Tumber , A., Harding, J. C. S., Krakowka, S., Ellis, J. A. & Hill, J. E. (2009) Development and validation of a SYBR green real-time PCR for the quantification of porcine circovirus type 2 in serum, buffy coat, feces, and multiple tissues. *Vet Microbiol*, **133**, 23–33.

Meulenberg, J.J., Petersen-den Besten, A., De Kluyver, E.P., Moormann, R.J., Schaaper, W.M. & Wensvoort, G. (1995) Characterization of proteins encoded by ORFs 2 to 7 of Lelystad virus. *Virology*, **206**(1), 155-163.

Mur, L., Gallardo, C., Soler, A., Zimmermman, J., Pelayo, V., Nieto, R., Sánchez-Vizcaíno, J.M. & Arias, M. (2013) Potential use of oral fluid samples for serological diagnosis of African swine fever. *Vet Microbiol*, **165**(1-2), 135-139.

Myers, K.P., Olsen, C.W. & Gray, G.C. (2007) Cases of swine influenza in humans: a review of the literature. *Clin Infect Dis*, **44**(8), 1084-1088.

Naing, L., Winn, T. & Rusli, B.N. (2006) Practical Issues in Calculating the Sample Size for Prevalence Studies. *Arch Orofacial Sci*, **1**, 9-14.

Nelson, M.I., Detmer, S.E., Wentworth, D.E., Tan, Y., Schwartzbard, A., Halpin, R.A., Stockwell, T.B., Lin, X., Vincent, A.L., Gramer, M.R. & Holmes, E.C. (2012) Genomic reassortment of influenza A virus in North American swine, 1998-2011. *J Gen Virol*, **93**(Pt 12), 2584-2589.

Nelson, M.I., Stratton, J., Killian, M.L., Janas-Martindale, A. & Vincent, A.L. (2015a) Continual reintroduction of human pandemic H1N1 influenza A viruses into swine in the United States, 2009 to 2014. *J Virol*, **89**(12), 6218-6226.

Nelson, M.I., Viboud, C., Vincent, A.L., Culhane, M.R., Detmer, S.E., Wentworth, D.E., Rambaut, A., Suchard, M.A., Holmes, E.C. & Lemey, P. (2015b) Global migration of influenza A viruses in swine. *Nat Commun*, 27, 6:6696.

Nelson, M.I., Wentworth, D.E., Culhane, M.R., Vincent, A.L., Viboud, C., LaPointe, M.P., Lin, X., Holmes, E.C. & Detmer S.E. (2015c) Introductions and evolution of human-origin seasonal influenza A viruses in multinational swine populaions. *J Virol*, **88**(17), 10110-10119.

OIE. (2013) Principles and methods of validation of diagnostic assays for infectious diseases. In *2013 Terrestrial Manual*. http://www.oie.int/fileadmin/Home /eng/Health_standards/tahm/1.01.05_VALIDATION.pdf, accessed on 10 December 2015.

OIE. (2015) Porcine reproductive and respiratory syndrome. In *2015 Terrestrial Manual* http://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/2.08.07_PRRS.pdf, access on 10 December 2015.

Olsen, C.W. (2012) Sampling considerations pertinent to the detection of analytes in swine oral fluids (Doctoral dissertation). Retrieved from Iowa State University, Ames, Iowa, http://lib.dr.iastate.edu/cgi/viewcontent.cgi?article=3639&context=etd.pdf, accessed on 13 December 2015 Paper 12632.

Olsen, C.W. (2002) The emergence of novel swine influenza viruses in North America. *Virus Res*, **85**(2), 199-210.

Olsen C.W., Brown I.H., Easterday B.C. & Van Reeth K. (2006). Swine Influenza. In: Diseases of Swine, Straw B.E., Zimmerman J.J., Taylor D.J. & D'Allaire S., eds. Ames, Iowa State University Press, 469–482.

Olvera, A., Cortey, M. & Segales, J. (2007) Molecular evolution of porcine circovirus type 2 genomes: phylogeny and clonality. *Virology*, **357**(2), 175-185.

Opriessnig, T. & Halbur, P.G. (2012) Concurrent infections are important for expression of porcine circovirus associated disease. *Virus Res*, **164**(1-2), 20-32.

Opriessnig, T. & Langohr, I. (2013) Current state of knowledge on porcine circovirus type 2-associated lesions. *Vet Pathol*, **50**(1), 23- 38.

Opriessnig, T., Gimenez-Lirola, L.G. & Halbur, P.G. (2011) Polymicrobial respiratory disease in pigs. *Anim Health Res Rev*, **12**(2), 133-148.

Opriessnig, T., McKeown, N.E. & Zhou, E.M. (2006) Genetic and experimental comparison of porcine circovirus type 2 (PCV2) isolates from cases with and without PCV2-associated lesions provides evidence for differences in virulence. *J Gen Virol*, **87**, 2923–2932.

Opriessnig, T., Meng, X.J. & Halbur, P.G. (2007) Porcine circovirus type 2 associated disease: update on current terminology, clinical manifestations, pathogenesis, diagnosis, and intervention strategies. *J Vet Diagn Invest*, **19**(6), 591-615.

Opriessnig, T., Patterson, A.R. & Meng, X.J. (2009) Porcine circovirus type 2 in muscle and bone marrow is infectious and transmissible to naive pigs by oral consumption. *Vet Microbiol*, **133**, 54–64.

Otake, S., Dee, S., Corzo, C., Oliveira, S. & Deen, J. (2010) Long-distance airborne transport of infectious PRRSV and Mycoplasma hyopneumoniae from a swine population infected with multiple viral variants. *Vet Microbiol*, **145**(3-4), 198-208.

Palese, P. (2004) Influenza: old and new threats. *Nat Med*, **10**(12 Suppl), S82-87.

Panyasing, Y., Goodell, C.K., Gimenez-Lirola, L., Kittawornrat, A., Wang, C., Schwartz, K.J. & Zimmerman, J.J. (2013) Kinetics of influenza A virus nucleoprotein antibody (IgM, IgA, and IgG) in serum and oral fluid specimens from pigs infected under experimental conditions. *Vaccine*, **31**(52), 6210-6215.

Panyasing, Y., Goodell, C., Kittawornrat, A., Wang, C., Levis, I., Desfresne, L., Rauh, R., Gauger, P.C., Zhang, J., Lin, X., Azeem, S., Ghorbani-Nezami, S., Yoon, K.J. & Zimmerman, J. (2014) Influenza A Virus Surveillance Based on Pre-Weaning Piglet Oral Fluid Samples. *Transbound Emerg Dis*.

Pearce, N. (2004) Effect measures in prevalence studies. *Environ Health Perspect*, **112**(10), 1047-1050.

Pfaffl, M.W. (2001) A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res*, **29**(9), e45.

Prickett, J.R., Cutler, S., Kinyon, J.M., Naberhaus, N., Stensland, W., Yoon, K.J. & Zimmerman, J.J. (2010a) Stability of porcine reproductive and respiratory syndrome virus and antibody in swine oral fluid. *J. Swine Health and Prod***, 18**(4), 187-195.

Prickett, J.R. & Zimmerman, J.J. (2010b) The development of oral fluid-based diagnostics and applications in veterinary medicine. *Anim Health Res Rev*, **11**(2), 207-216.

Prickett, J., Simer, R., Christopher-Hennings, J., Yoon, K.J., Evans, R.B. & Zimmerman, J.J. (2008) Detection of Porcine reproductive and respiratory syndrome virus infection in porcine oral fluid samples: a longitudinal study under experimental conditions. *J Vet Diagn Invest*, **20**(2), 156-163.

Rajao, D.S., Anderson, T.K., Gauger, P.C. & Vincent, A.L. (2014) Pathogenesis and vaccination of influenza A virus in swine. *Curr Top Microbiol Immunol*, **385**, 307-326.

Raymaekers, M., Smets, R., Maes, B. & Cartuyvels, R. (2009) Checklist for optimization and validation of real-time PCR assays. *J Clin Lab Anal*, **23**(3), 145-151.

Reid, A.H., Fanning, T.G., Hultin, J.V. & Taubenberger, J.K. (1999) Origin and evolution of the 1918 "Spanish" influenza virus hemagglutinin gene. *Proc Natl Acad Sci U S A*, **96**(4), 1651-1656.

Richt, J.A., Lager, K.M., Janke, B.H., Woods, R.D., Webster, R.G. & Webby, R.J. (2003) Pathogenic and antigenic properties of phylogenetically distinct reassortant H3N2 swine influenza viruses cocirculating in the United States. *J Clin Microbiol*, **41**(7), 3198-3205.

Romagosa, A., Allerson, M., Gramer, M., Joo, H.S., Deen, J., Detmer, S. & Torremorell, M. (2011) Vaccination of influenza a virus decreases transmission rates in pigs. *Vet Res*, **42**, 120.

Romagosa, A., Gramer, M., Joo, H.S. & Torremorell, M. (2012) Sensitivity of oral fluids for detecting influenza A virus in populations of vaccinated and non-vaccinated pigs. *Influenza Other Respir Viruses*, **6**(2), 110-118.

Rosell, C., Segalés, J., Plana-Duran, J., Balasch, M., Rodríguez-Arrioja, G.M., Kennedy, S., Allan, G.M., McNeilly, F., Latimer, K.S. & Domingo, M. (1999) Pathological, immunohistochemical, and in-situ hybridization studies of natural cases of post- weaning multisystemic wasting syndrome (PMWS) in pigs. *J. Comp. Pathol*, **120**(1), 59–78.

Rosell, C., Segales, J., Rovira, A. & Domingo, M. (2000) Porcine circovirosis in Spain. *Vet Rec*, **146**(20), 591-592.

Rossen, L., Norskov, P., Hoimstrom, K. & Rasmussen, O. F. (1992) Inhibition of PCR by components of food samples, microbial diagnostic assays and DNA-extraction solutions.

International journal of food microbiology, **17**, 37-45.

Rossow, K.D., Collins, J.E., Goyal, S.M., Nelson, E.A., Christopher-Hennings, J. & Benfield, D.A. (1995) Pathogenesis of porcine reproductive and respiratory syndrome virus infection in gnotobiotic pigs. *Vet Pathol*, **32**(4), 361-373.

Saitou, N. & Nei, M. (1987) The Neighbor-joining Method: A New Method for Reconstructing Phylogenetic Trees. *Mol. Biol. Evol*. **4**(4), 406-425.

Schultz-Cherry, S., Olsen, C.W. & Easterday, B.C. (2013) History of Swine influenza. *Curr Top Microbiol Immunol*, **370**, 21-28.

Seddon, Y.M., Guy, J.H. & Edwards, S.A. (2012) Optimising oral fluid collection from groups of pigs: effect of housing system and provision of ropes. *Vet J*, **193**(1), 180-184.

Segalés, J., Allan, G.M. & Domingo, M. (2005) Porcine circovirus diseases. *Anim Health Res Rev*, **6**(2), 119-142.

Segalés, J., Rosell, C. & Domingo, M. (2004) Pathological findings associated with nat- urally acquired porcine circovirus type 2 associated disease. *Vet. Microbiol*, **98**(2), 137–149 .

Segalés, J., Piella, J., Marco, E., Mateu-de-Antonio, E.M., Espuna, E. & Domingo, M. (1998) Porcine dermatitis and nephropathy syndrome in Spain. *Vet Rec*, **142**(18), 483-486.

Shope, R. E. & Lewis, P. A. 1931 Swine influenza. *J. Exp. Med*. 54, 349-359.

Sibila, M., Pieters, M., Molitor, T., Maes, D., Haesebrouck, F. & Segales, J. (2009) Current perspectives on the diagnosis and epidemiology of Mycoplasma hyopneumoniae infection. *Vet J*, **181**(3), 221-231.

Sponseller, B.A., Strait, E., Jergens, A., Trujillo, J., Harmon, K., Koster, L., & et al. (2010) Influenza A pandemic (H1N1) 2009 virus infection in domestic cat. *Emerg Infect Dis,* **16**(3): 534-537

Strait, E.L., Madsen, M.L., Minion, F.C., Christopher-Hennings, J., Dammen, M., Jones, K.R., & Thacker E.L. (2008) Real-time PCR assays to address genetic diversity among strains of *Mycoplasma hyopneumoniae. J Clin Microbiol,* 46:2491-2498.

Swenson, S.L., Hill, H.T., Zimmerman, J.J., Evans, L.E., Landgraf, J.G., Wills, R.W., Sanderson, T.P., McGinley, M.J., Brevik, A.K., Ciszewski, D.K. & et al. (1994) Excretion of porcine reproductive and respiratory syndrome virus in semen after experimentally induced infection in boars. *J Am Vet Med Assoc*, **204**(12), 1943-1948.

Tamura, K., Nei, M. & Kumar, S. (**2004**) Prospects for inferring very large phylogenies by using the neighbor-joining method. *Proceedings of the National Academy of Sciences* (*USA*), **101**:11030-11035.

Tamura, K., Stecher, G., Peterson, D., Filipski, A. & Kumar, S. (2013) MEGA6: Molecular

Evolutionary Genetics Analysis Version 6.0. *Mol. Biol. Evol*, **30**(12), 2725–2729.

Taubenberger, J.K., Reid, A.H., Janczewski, T.A. & Fanning, T.G. (2001) Integrating historical, clinical and molecular genetic data in order to explain the origin and virulence of the 1918 Spanish influenza virus. *Philos Trans R Soc Lond B Biol Sci*, **356**(1416), 1829-1839.

Terpstra, C., Wensvoort, G. & Pol, J.M. (1991) Experimental reproduction of porcine epidemic abortion and respiratory syndrome (mystery swine disease) by infection with Lelystad virus: Koch's postulates fulfilled. *Vet Q*, **13**(3), 131-136.

Tischer, I., Peters, D., Rasch, R. & Pociuli, S. (1987) Replication of porcine circovirus: induction by glucosamine and cell cycle dependence. *Arch Virol*, **96**(1-2), 39-57.

Tischer, I., Gelderblom, H., Vettermann, W. & Koch, M.A. (1982) A very small porcine virus with circular single-stranded DNA. *Nature*, **295**, 64-66.

Tischer, I., Rasch, R. & Tochtermann, G. (1974) Characterization of papovavirus-and picornavirus-like particles in permanent pig kidney cell lines. *Zentralbl Bakteriol Orig A*, **226**(2), 153-167.

Tong, S., Li, Y., Rivailler, P., Conrardy, C., Castillo, D.A., Chen, L.M., Recuenco, S., Ellison, J.A., Davis, C.T., York, I.A., Turmelle, A.S., Moran, D., Rogers, S., Shi, M., Tao, Y., Weil, M.R., Tang, K., Rowe, L.A., Sammons, S., Xu, X., Frace, M., Lindblade, K.A., Cox, N.J., Anderson, L.J., Rupprecht, C.E. & Donis, R.O. (2012) A distinct lineage of influenza A virus from bats. *Proc Natl Acad Sci U S A*, **109**(11), 4269-4274.

Van Reeth, K. & Ma, W. (2013) Swine influenza virus vaccines: to change or not to change-that's the question. *Curr Top Microbiol Immunol*, **370**,173-200.

Van Reeth, K., Brown, I.H. & Olsen, C.W. (2012) Influenza virus. In Zimmerman (Ed) *Diseases of Swine*, 10th Edn (2036-2095). Ames, IA: John Wiley & Sons, Inc.

Vincent, A.L., Ma, W., Lager, K.M., Janke, B.H. & Richt, J.A. (2008) Swine influenza viruses a North American perspective. *Adv Virus Res*, **72**, 127-154.

Vosloo, W., Morris, J., Davis, A., Giles, M., Wang, J., Nguyen, H.T., Kim, P.V, Quach, N.V., Le, P.T., Nguyen, P.H., Dang, H., Tran, H.X., Vu, P.P., Hung, V.V., Le, Q.T., Tran, T.M., Mai, T.M., Le, Q.T. & Singanallur, N.B. (2015) Collection of oral fluid using cotton ropes as a sampling method to detect foot-and-mouth disease virus infection in pigs. *Transbound Emerg Dis*, **62**(5), 71-75.

Wagstrom, E.A., Chang, C.C., Yoon, K.J. & Zimmerman, J.J. (2001) Shedding of porcine reproductive and respiratory syndrome virus in mammary gland secretions of sows. *Am J Vet Res*, **62**(12), 1876-1880.

Wang, F., Guo, X., Ge, X., Wang, Z., Chen, Y., Cha, Z. & Yang, H. (2009) Genetic variation analysis of Chinese strains of porcine circovirus type 2. *Virus Res*, **145**(1), 151-156.

Webby, R.J., Swenson, S.L., Krauss, S.L., Gerrish, P.J., Goyal, S.M. & Webster, R.G. (2000) Evolution of swine H3N2 influenza viruses in the United States. *J Virol*, **74**(18), 8243-8251.

Webster, R.G. & Govorkova, E.A. (2006) H5N1 influenza--continuing evolution and spread. *N Engl J Med*, **355**(21), 2174-2177.

Webster, R.G. (2002) The importance of animal influenza for human disease. *Vaccine*, **20 Suppl 2**, S16-20.

Wensvoort, G. (1993) Lelystad virus and the porcine epidemic abortion and respiratory syndrome. *Vet Res*, **24**(2), 117-124.

Wensvoort, G., Terpstra, C., Pol, J.M., ter Laak, E.A., Bloemraad, M., de Kluyver, E.P., Kragten, C., van Buiten, L., den Besten, A., Wagenaar, F. & et al. (1991) Mystery swine disease in The Netherlands: the isolation of Lelystad virus. *Vet Q*, **13**(3), 121-130.

West, K.H., Bystrom, J.M., Wojnarowicz, C., Shantz, N., Jacobsen, M., Allan, G.M., Haines, D.M., Clark, E.G., Krakowka, S.J., McNeilly, F., Konoby, C., Martin, K. & Ellis, J.A. (1999) Myocarditis and abortion associated with intrauterine infection of sows with porcine cirocovirus 2. *J Vet Diagn Invest*, **11**, 530-532.

Wills, R.W., Zimmerman, J.J., Yoon, K.J., Swenson, S.L., McGinley, M.J., Hill, H.T., Platt, K.B., Christopher-Hennings, J. & Nelson, E.A. (1997) Porcine reproductive and respiratory syndrome virus: a persistent infection. *Vet Microbiol*, **55**(1-4), 231-240.

Woolley, L.K., Fell, S., Gonsalves, J.R., Walker, M.J., Djordjevic, S.P., Jenkins, C. & Eamens, G.J. (2012) Evaluation of clinical, histological and immunological changes and qPCR detection of *Mycoplasma hyopneumoniae* in tissues during the early stages of mycoplasmal pneumonia in pigs after experimental challenge with two field isolates. *Vet Microbiol,* **161**(1-2):186-95.

Yedidag, E.N., Koffron, A.J., Mueller, K.H., Kaplan, B., Kaufman, D.B., Fryer, J.P., Stuart, F.P. & Abecassis, M. (1996) Acyclovir triphosphate inhibits the diagnostic polymerase chain reaction for cytomegalovirus. *Transplantation*, **62**(2), 238-242.

Zhou, Y.J., Yu, H., Tian, Z.J., Liu, J.X., An, T.Q., Peng, J.M., Li, G.X., Jiang, Y.F., Cai, X.H., Xue, Q., Wang, M., Wang, Y.F. & Tong, G.Z. (2009) Monoclonal antibodies and conserved antigenic epitopes in the C terminus of GP5 protein of the North American type porcine reproductive and respiratory syndrome virus. *Vet Microbiol*, **138**(1-2), 1-10.

Zimmerman, J.J., Benfield, D.A., Dee, S.A., Murtaugh, M.P., Stadejek, T., Stevenson, G.W. & Torremorell, M. (2012) Porcine Reproductive and Respiratory Syndrome Virus (Porcine Arterivirus), In Zimmerman (Ed) *Diseases of Swine*, 10th Edn (1675-1777). Ames, IA: John Wiley & Sons, Inc.